

Tough road to LHC is worth taking

There are problems in getting collaboration on the LHC off the ground, but it should be possible to overcome them, to the benefit of all concerned.

THE design and construction of the proposed Large Hadron Collider (LHC) at the European Laboratory for Particle Physics (CERN) in Geneva, Switzerland, will be a tough technical challenge. But the history of particle physics provides strong grounds for believing the challenge can be met.

The same cannot quite be said of the challenge faced by those now working to secure US backing for the LHC project. The US Congress has never before been asked to provide the kind of funding required for LHC — perhaps \$500 million over 8–10 years — to help build a scientific facility abroad. There are, however, grounds for believing that US backing will eventually be secured.

The existing CERN partners are firmly locked into their collaboration by international treaty. But Congress alone will decide what the United States will contribute, and it will do so each year and every year. It will not be bound by its own previous declarations, still less by the views of US government officials.

High-energy physicists are justifiably encouraged that negotiations between CERN and the US Department of Energy (DOE) have reached the stage at which both sides can publicly agree on the amount of US support they are discussing (see *Nature* 380, 369; 1996). But it is hardly time to uncork the champagne.

The United States had been hinting for some time at a \$400-million contribution. The Europeans had hoped for up to twice as much: they reasoned that American scientists would use the machine more than any other national group. The real cost of the project, including the contribution of European physicists whose work will not be billed to it, is considerably higher than the officially stated project cost of \$3 billion.

After meetings in Washington this past winter, at which US budget problems loomed large, the United States suggested a figure as a basis for negotiation — \$450 million, plus or minus \$50 million, from the DOE and an additional \$80 million from the National Science Foundation. Christopher Llewellyn Smith, the director of CERN, took this back to a CERN council meeting, whose members swallowed hard, looked reality in the face, and reluctantly agreed that the figure could form a basis for negotiation.

The two sides have given themselves until the end of the year to come up with a deal. That process in itself could be endangered by the US presidential elections in November: Martha Krebs, assistant energy secretary and head of the US negotiating team, is out of a job if President Bill Clinton loses. But if things go smoothly, an agreement could be accepted by the CERN Council and put to Congress at the beginning of next year. An effort will then be made to pass an authorization bill — an approval, in principle, of future expenditure — to show that Congress supports the project. Such an endorsement would still leave Congress appropriations committees to decide, each year, whether LHC support should continue inside a budget that will no doubt grow even tighter and more difficult every time.

The influential High Energy Physics Advisory Panel (HEPAP) said, in 1994, that the United States could afford the contribution in the peak period of 1999–2004 because two major high-energy physics construction projects in the United States will be completed by 1998. But the high-energy physics budget of \$650 million is normally lumped in with a nuclear physics budget of \$350 million, and the latter needs to grow sharply to enable the full opera-

tion of two other facilities that will be completed shortly. And the combined budget of \$1 billion could be severely curtailed by budget pressure at DOE; one set of figures has it collapsing to \$760 million by 1999.

Still, where would we be without hope? US physicists need the LHC to keep up with the Europeans and the project will benefit greatly from US technical expertise. HEPAP retains considerable prestige, and leaders of both parties in Congress will pay attention to its advice. James Sensenbrenner (Republican, Wisconsin), who is likely to be the next chair of the Science Committee in the House of Representatives, is a strong internationalist, as are most of the congressional leaders who oversee this programme on both sides of the aisle, in both House and Senate.

That could change, of course. A number of these leaders will retire this year. It will be an immense challenge for the particle physics community to ensure that their successors understand and support what is being planned at CERN. They must also understand what is at stake, which is the credibility of the United States as a reliable partner in a field of science where future progress will depend on international collaboration. □

Private capital is no answer

The British government should admit that its proposed solution for equipment funding in universities is mistaken.

ONE sign of strong government is the willingness to admit mistakes and the British government has an opportunity to salvage a small part of its increasingly fragile reputation. At issue is the deteriorating state of the scientific facilities used for teaching and research in British universities. The mistake is the belief that universities should be able to find private capital to fund such facilities.

The fallacy has already made a serious dent in the government's reputation as a supporter of science. In the 1995–96 budget figures, released in February, officials pointed out proudly that spending on science through the research councils will be roughly in line with the anticipated rate of inflation. But this welcome gain has been almost wiped out by a decision to impose a significant cut on capital spending for equipment by universities.

If these cuts are sustained, academic morale will inevitably drop even further. As a report published this week points out (see page 572), overcrowding and under-investment are already threatening the ability of universities to meet their research responsibilities. Private capital is not the solution to research equipment needs. Universities cannot borrow such money, being unable to provide a guarantee of a sufficient income stream to meet repayments. Industry — like the research charities — says it is the government's role to see that university infrastructure is adequately financed.

Finding a solution will not be easy. But the amount of money involved could probably be met by careful surgery elsewhere, while the needs it would meet are essential for the health of British science. Government ministers seem to have begun to recognize the dangers of the course they are on. The next step will be to turn this into remedial action. □



Mobile telephones ring out changes for radioastronomy frequencies

Munich. European radioastronomers agreed last week to discuss a proposal that transmitting beacons should be set up next to their receivers to instruct mobile telephone users in their vicinity to switch from radioastronomy frequencies. The proposal had been made by the satellite company Iridium, a subsidiary of Motorola, to allow protected radiofrequencies to be shared by astronomers and mobile telephone users.

Meeting at the Nuffield Radioastronomy Laboratory at Jodrell Bank in Cheshire, England, the Committee on Radioastronomy Frequencies (CRAF), an *ad hoc* committee of the European Science Foundation, said it would be prepared to consider the 'beacon option' when it received from Iridium a more detailed technical specification.

But Jim Cohen, the chairman of CRAF, and a reader in radioastronomy at the University of Manchester, warned that European radioastronomers would wish to see a beacon functioning properly before making a firm agreement, and that it might be necessary to modify receivers to reduce sensitivity. For this reason, he says, CRAF will insist on similar compromises from Iridium to avoid interference from satellite signals returning to Earth, which at peak times spill over into radioastronomical frequencies.

Iridium is one of several companies planning to launch satellites for the global mobile communications market, but its unique design — using the same frequency band (1610.0–1626.5 MHz) for both uplinks and downlinks — threatens much more interference.

Satellite companies were allocated this frequency range by the International Telecommunication Union (ITU) at its World Radio Conference in 1992. Because it overlaps with the 1610.0–1613.8 MHz frequency range, which is protected for radioastronomy observations of the hydroxyl radical, a footnote to the allocation states that "harmful interference should not be caused to radioastronomy".

Mobile satellite companies will use the 1610.6–1626.5 MHz frequency band for sending signals from handsets to the satellite (the uplink) and accept that they must provide exclusion zones around radiotelescopes. It is only the mechanism for establishing such an exclusion zone that remains controversial.

Radioastronomers have up to now been reluctant to accept Iridium's beacon option.

But they now agree that having a transmitter beacon close to receivers may not cause the technical problems they had initially feared. Nevertheless, Cohen says, it may cause other problems. In the Netherlands, for example, radio-observatories are placed in areas where no form of transmission is allowed. Radioastronomers would prefer mobile satellite companies to build a system into handsets that would allow their position to be located by satellite, an option that some companies, such as Global Star, have already accepted.

But Peter Fischer, a representative of Iridium based in Bonn, says that the beacon option is technically superior. "Relying on the less precise satellite location of handsets would render the exclusion zone unneces-

sary for mobile telephone users. According to Cohen, this would effectively halve the time available for observations, already too limited for the liking of scientists, and would make certain observations technically impossible. Particularly affected would be the Nançay Radioastronomy Observatory south of Paris, which uses this frequency for a large fraction of its observations.

Fischer says Iridium is ready and willing to try to understand the needs of the radioastronomers. Indeed, the company will meet scientists, as well as British radiofrequency licensing authorities, in London next week to take negotiations further forward.

In particular, he would like to understand why European radioastronomers would not be happy with an agreement similar to the

memorandum of understanding signed with the US National Radioastronomy Observatory in 1994. This guarantees four hours of zero radiointerference per day, during times of low user demand. It also provides equipment to the observatory to allow scientists to use the pulse gaps.

Both Fischer and Cohen are members of a CEPT (European Conference of Post and Telecommunications) project team which must complete a

report on the problem by October. Fischer says that a solution acceptable to both sides is certain to be found "because we all have to share the wavelengths". But the process will not be bloodless. CRAF intends to negotiate as determinedly as possible for its preferred option: no interference at all with radioastronomy frequencies.

Scientists fear that with the global mobile telephone system gaining momentum, the problem with interference will become increasingly worse. "The companies will just expand and expand," says Klaus Ruf of the Max Planck Institute for Radioastronomy in Bonn, who sees grave danger in giving in now to market pressures. Cohen points out that radioastronomers have only just solved a similar problem with the Russian Global Navigation Satellite System, GLONASS, which was launched in 1982. Three years ago, Russia agreed to make changes to the system to curb its massive interference with radioastronomy. But the changes will take ten years to complete. Cohen is committed to trying to avoid similar problems in the future.

Alison Abbott

See also page 572

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Crossed lines? The radio frequencies used by mobile phones and radioastronomers overlap.

sarily large," he says. It is also rather late in the day for a system redesign. The company is planning to start launching its battery of 66 satellites by the

end of the year, and already has an experimental licence to test the system in the United States.

The biggest potential for interference comes from the downlink, the connection between the satellite and the handset. This problem that applies only to Iridium, as other companies plan to use frequencies distant from radioastronomy frequencies. "The satellite will emit a lot of unwanted, polluting, signals, particularly at times of heavy load," says Cohen. This is where he and his colleagues believe that Iridium should compromise, by capping the number of subscribers using telephones at peak time to reduce interference. But such a solution is clearly not compatible with a commercial aim of maximizing revenue.

Radioastronomers are not prepared to accept Iridium's idea of time-sharing of frequencies, whereby they would be able to use only the gaps in the signal pulses left over by

Martin Bone/SPL

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US military tightens rules on DNA records

Washington. The US Department of Defense (DoD) has agreed to modify the rules governing the use of the DNA records it keeps on military personnel, in response to increasing public concern that the records could be misused.

The move comes as two US Marines — Lance Corporal John Mayfield and Corporal Joseph Vlacovsky — are due to be court-martialled in Hawaii next week for refusing to contribute a DNA sample to what is believed to be the world's largest gene bank (see *Nature* 379, 385; 1996).

The DoD, which says it has built up its DNA records solely to aid the identification

of troops killed in battle, now says that individuals can ask for their own record to be destroyed when they leave military service.

It also promises to destroy all such records itself after 50 years, rather than 75 years as it had previously promised, and has placed tight restrictions on the circumstances in which the information can be used for purposes other than identification of remains. But it still plans to continue the compulsory collection of DNA samples from soldiers and support personnel.

Eric Seitz, a civil rights lawyer representing the two Marines due to be court-martialled next week, claims that the DoD is

concerned that by not taking action over Mayfield and Vlacovsky, it would be "setting a precedent" that would encourage others to refuse to give the blood and tissue samples to the bank.

At a press conference in Washington last week, Mayfield and Vlacovsky said they had been unaware of the issues surrounding DNA testing when first asked to provide the samples. But they became suspicious when the DoD was unable to answer their questions about how the samples would be used.

Under a directive from Stephen Joseph, assistant secretary for health at DoD, DNA samples will now be used for purposes other than identifying remains only if the donor gives consent, or if the specimen is needed for the investigation of a serious crime.

Nevertheless, Wendy McGoodwin, director of the Council for Responsible Genetics (CRG), a lobby group which is supporting the Marines in a civil court action against DoD, dismisses the new directive as little more than window-dressing.

"We don't consider this to be a substantial alteration to the policy," says McGoodwin. The CRG is concerned that the one million records already contained in the DNA bank might eventually be used to support "genetic discrimination" inside and outside the military.

Seitz says that he welcomes the changes in the directive, while insisting that the civil action against the DoD will continue. But the Department of Defense itself hopes that its concessions will not only stifle the court challenge to its actions but also calm public concern about potential misuse of the gene bank.

Colin Macilwain

Court allows fossil fraudster to return

New Delhi. A move by Panjab University in Chandigarh, India, to get rid of its controversial geologist, Viswa Jit Gupta, has backfired, causing dismay in the academic community and considerable embarrassment to authorities. Only three days after his offer to leave "voluntarily" was accepted by the university senate last month and he was allowed to retire, Gupta, who had been accused of faking Himalayan fossils (see *Nature* 369, 698; 1994), was reinstated by order of the Punjab and Haryana high court.

The judges overturned the senate decision on the grounds that Gupta had written a second letter withdrawing his resignation. The court therefore permitted Gupta to return to his job. Ironically, one result of this unexpected development is that the fossil case has ceased to be a domestic university affair and has moved to the courtroom.

The recent events began soon after Gupta had learnt that the university had called a special meeting of its senate on 17 March on the orders of India's vice-president, K. R. Narayanan, who is also chancellor of the university. Suspecting that the meeting was planned to dismiss him, Gupta asked on 1 March for voluntary retirement, and demanded that the meeting be cancelled. When the vice-chancellor, T. N. Kapur, insisted on holding the meeting, Gupta sought the court's protection against any action by the senate, and sent the university another letter, withdrawing his earlier letter of resignation.

The court subsequently issued an order restraining the university from inflicting any "further punishment" on Gupta. As a result, the subsequent senate meeting could not take any action against him. Its only course of action was to accept his application for voluntary retirement and reject his second letter withdrawing his resignation. But Gupta appealed to the court again, arguing that the senate acted wrongly in rejecting his second letter — and won.

The fossil fraud was brought to the notice

of the scientific community seven years ago by John Talent of Australia (see *Nature* 338, 613; 1989), but the Panjab University took nearly five years to declare Gupta guilty of misconduct. Even then, he received only mild punishment: his annual salary increments were stopped, but he continued to teach students, while his co-workers who had helped to expose the fraud were harassed by Gupta and his supporters in the senate (see *Nature* 378, 227; 1995).

Embarrassed by Gupta's continued presence on the university payroll, as well as criticism in both the national and international press, Narayanan had called the senate meeting to "take steps to restore the fair name of the university". But the course of action taken by the senate, far from penalizing Gupta, have now turned out to his advantage.

K. S. Jayaraman

NASA unveils astrophysics missions

Washington. The US National Aeronautics and Space Administration (NASA) inaugurated its new line of Medium-class Explorer (MIDEX) missions for space physics and astrophysics last week with the selection of two spacecraft to be launched in 1999 and 2000.

Charles Bennett of NASA's Goddard Space Flight Center in Maryland leads the Microwave Anisotropy Probe (MAP) project, to study the cosmic microwave background to help understanding of large-scale structure in the Universe. The Imager for Magnetopause-to-Aurora Global Exploration (IMAGE), led by James Burch of the Southwest Research Institute in San Antonio, Texas, will image the interaction of the Earth's magnetosphere with the solar wind.

The order of launch and exact launch dates are still to be decided. Both missions will receive money for develop-

ment studies, with the final go-ahead for the first mission expected in about a year. Development costs for MIDEX missions are capped at \$70 million, not counting the launch vehicle, mission operations and data analysis.

The agency's 'New Millennium' programme, planned to develop advanced space technology, announced another flight project last week to test a new kind of Earth science sensor in orbit. The Advanced Land Imager instrument, to be built by a team led by the Massachusetts Institute of Technology's Lincoln Laboratory, will be sensitive in visible and short-wave infrared wavelengths.

The device is meant to improve on existing Landsat spectrometers in nearly every way — higher resolution, lower mass and lower cost. The test mission's overall cost, including launch, is being capped at \$90 million. **Tony Reichhardt**

Support for teaching 'could boost university research'

London. A lack of investment has left British universities unable to meet the research demands being placed upon them, according to a report published this week by the National Academies Policy Advisory Group*, a body established jointly by Britain's leading academies of science, engineering, medicine, humanities and social science.

The group says that better support for university teaching would help the research side to develop properly by enabling universities to focus on their individual strengths. Indeed, it recommends that the funding councils responsible for allocating funds to universities should earmark about £50 million a year for professional development and teaching, using money drawn from elements of current research funding.

Its report adds to the mounting pressure on the UK government to increase capital spending on universities. But little significant government action is likely to be taken until after the Dearing Committee on Higher Education reports in 1997.

While pressing the government to recognize the urgent capital needs of universities, especially for libraries, modern equipment and building maintenance, the report stops short of demanding more money from the government for universities.

"We saw no point in simply asking the government for more money," says David Harrison, master of Selwyn College, Cambridge, and chairman of the working group. Rather, the report recommends that existing funds be reallocated to make the best use of resources, including the concentration of research funding.

The £50 million allocated to teaching development, for example, could come from funds used to support research gaining a score of two or less (out of five) in the funding councils' regular Research Assessment Exercise. This proposal is an attempt to curb 'mission drift' – the tendency for individual universities to pursue funds for research even if they lack the necessary resources to carry it out effectively.

By making this recommendation, the report takes a wider perspective than expected. "We are recommending a shift in funding from low quality research to high quality teaching," says Harrison. He acknowledges that some universities will gain and some will lose under such a shift, but believes that it would help each university to concentrate on what it does best.

Alistair MacFarlane, principal of Heriot-

Watt University, Edinburgh, and a member of NAPAG, says the working party endorsed the principle that engaging in research requires "some component of scholarship and research" while recognizing that the highest level of research requires a concentration of funding. The working group had aimed to reconcile the requirements of excellence in teaching and research.

Recent government cuts in university spending have put both teaching and research under great strain. But the pressure on teaching has grown enormously along

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Class act: high quality teaching should gain funds from low quality research, report says.

with the rise in student/staff ratios. On average across all disciplines, they have risen from 11 to 15 between 1989 and 1994.

"Teaching should have a higher status and not be regarded as a poor relation to research," Harrison adds. By seeking to relieve teaching pressure, the proposal specifically addresses the need for teachers in universities to update and refresh themselves through scholarly activity.

The proposal also addresses the anxiety of universities that the present funding methodology is unbalanced. Michael Powell, senior administrative officer at the Committee of Vice-Chancellors and Principals, says that "at present there is an element in favour of promoting excellence in research, but nothing to enhance the status of teaching".

David Triesman, general secretary of the Association of University Teachers, says that he welcomes "every authoritative voice raised in the interest of funding high quality research because the reality is that the cuts in capital funding and revenue this year threaten to force the UK to the very bottom of the OECD league table".

The report does not recommend that research funding be shifted from low rated research to higher rated research. But Harrison believes that the group's proposals would improve the quality of research by concentrating research funding. **Ruth Bell**

Radioastronomers fight broadcasters' challenge in Italy

Munich. Italy's national research council, the CNR, has called on the government to protect the interests of Italian radioastronomers, who have effectively lost the use of a scientifically important waveband to commercial radio stations.

The post and telecommunications ministry last year introduced new regulations which, for the first time, allow radio stations access to the 1660–1670 MHz frequency, after they had been excluded from their previous frequency band following its reallocation to mobile telephone networks.

But this new frequency band is used by radioastronomers to measure emissions from galaxies, quasars and galactic sources such as molecular clouds. In compliance with international agreements, the new regulations give radioastronomers priority over the frequencies, and in principle licences are issued to radio stations only on condition that they immediately switch off if their broadcasts interfere with radioastronomy measurements.

And interfere they do. Lucia Padrielli, director of the CNR Institute of Radioastronomy in Bologna, says emissions from at least 20 different transmitters have been detected at the institute's radiotelescope site, and that strong pop music from these transmitters completely drowns out the faint signals from space.

But she says that complaints to the authorities are processed so slowly that it can take several months for transmitters to clear the wavebands. By this time, she says, "other stations have been given licences to take their place". Around a quarter of the institute's working time involves this frequency, she says.

Goliardo Tomassetti, a researcher at the institute, complains that no-one in Italy appears willing to take responsibility for the problem. Local licensing authorities say they act under instructions from the central ministry for post and telecommunications.

The ministry, in turn, says the interests of radioastronomers are protected by the law giving them priority rights to the frequencies. But commercial radio stations are now preparing to challenge this law, claiming the right to broadcast freely. The CNR is preparing a counterclaim.

The CNR last week mounted a major publicity campaign aimed at forcing the government to solve the current problem. If the government is let off the hook on this issue, says Tomassetti, it will have little incentive to take action to protect other frequencies that are important to radioastronomers. "It could be the end of radioastronomy in our country," he says.

Alison Abbott

*NAPAG is a body set up jointly by the British Academy, the Conference of Medical Royal Colleges, the Royal Academy of Engineering and the Royal Society. Copies of the report, *Research Capability of the University System*, are available from the publications department of the Royal Society, price £20.00.

Analysis of US budget plan confirms large cuts ahead

Washington. President Bill Clinton's plan to balance the US federal government's budget by the year 2002 would cut real spending on non-military research and development (R&D) programmes at the Department of Energy by 18 per cent, according to an assessment by the American Association for the Advancement of Science (AAAS) released this week. The research budget of the National Aeronautics and Space Administration would be cut by 17 per cent.

Overall spending on non-military R&D would fall by 11.7 per cent by the end of that period, says the AAAS. Its conclusions on the Clinton budget are less severe for 2002 than those of a similar assessment the AAAS carried out last year of the congressional budget plan, which would have cut non-defence R&D by 33 per cent. But Clinton's proposal brings spending down very sharply by the year 2000, when it would have fallen by 18 per cent from last year's level, according to the AAAS assessment.

Over the six-year period, the Department of the Interior — whose largest research component is the US Geological Survey — would be the hardest hit of the major government departments and agencies, with a real cut of 28 per cent. In contrast, the National Institutes of Health and the Environmental Protection Agency would fare best under the plan, with the research budgets of both holding stable until 2002.

"If these projections are actually realized, research in universities, federal laboratories and industry will face a potentially dire situation," says Al Teich, director of policy at the AAAS.

The AAAS, whose detailed assessments of budget information are intended to extract information about projected science spending, says that the 1997 budget proposed by Clinton last month would increase non-defence R&D by 3.3 per cent. It would then fall by about 5 per cent or so for each of the subsequent three years, before recovering in 2001 and 2002.

But an AAAS statement points out that many analysts believe "that the increases proposed for 2001 and 2002 are unrealistic because they are contingent upon economic forecasts that are overly optimistic". Both the Clinton and congressional plans hit research and development hard, as they assume that the government will cut taxes, maintain entitlements and balance the budget entirely by cutting back the so-called 'discretionary' spending approved each year by Congress. Teich points out that with R&D consuming one-seventh of all discretionary spending, it cannot escape severe cuts if the budget is balanced in this way.

Officials at NASA and the Department of Energy have already played down the significance of Clinton's future year projections, saying that they do not reflect policy judgements. But Teich warns the community against ignoring them. "You have to take them seriously — just as you had to take the Congressional budget resolution seriously — and work to ensure that they don't become a reality." **Colin Macilwain**

Funds fall behind demand for brain research grants

London. The growth in popularity of the Human Frontier Science Program (HFSP), which supports international scientific collaboration in research on brain functions and molecular approaches to the study of biological functions, seems to be confirmed by the fact that applications by young scientists for two-year research fellowships have increased by 19 per cent since last year.

At the same time, in announcing the details of its 1996 awards, HFSP officials say that the steady increase in the number of high-quality applications for awards over the past five years is causing concern among the trustees of the programme that many excellent applications are having to be turned down because of lack of funds.

"The increase in applications reflects the shortage of fellowships around the world," says Pierre Chambon of the Louis Pasteur University in Strasbourg, who is chairman of the HFSP's Council of Scientists, which supervises the scientific direction of its programme.

Chambon argues that the low success rate — only 10 per cent of applications for research awards, and 19 per cent of those for fellowships, are successful — adds to the prestige of the programme, whose current budget is US\$46 million a year. He would like to see the size of contributions to the programme increased; at present, about 80 per cent of the programme's income comes from Japan, while Europe and the United States each contribute about 10 per cent.

Forty-five research grants have been awarded in the current funding round, 14 in brain research and 31 in molecular studies. An important feature of the programme is that each grant is awarded to at least two teams from different countries. Among the 45 grants, 39 have at least one scientist working in the United States and 27 have at least one scientist working in Japan.

Responding to the needs of the international neuroscience community, HFSP has launched a programme of workshops on topical scientific issues in brain function and biological function at the molecular level. The first of these, "Coincidence Detection in the Nervous System", was held in Strasbourg in 1995. The proceedings form the first publication in a series aimed at specialists, postdocs and students. Ruth Bell

Quebec challenges blood inquiry

Ottawa. Quebec's government, which is committed to the separation of the province from Canada and achieving the status of an independent country, has filed a notice in federal court claiming that the federal inquiry into the supply to haemophiliacs of HIV-contaminated blood in the mid-1980s is unconstitutional.

Serge Kronstrom, a lawyer representing the provincial government, claimed in a court submission last week that a federal commission of inquiry "does not have the jurisdiction to investigate the affairs of the government of a province". As a result, he argued that the commission of inquiry, set up under Mr. Justice Horace Krever, acted outside its jurisdiction in issuing more than 300 preliminary notices of 'potential wrongdoing' (see *Nature* 379, 479; 1996).

But Robert Martin, professor of constitutional law at the University of Western Ontario, disagrees, arguing that the Canadian constitution divides only law-making

authority between the federal government and the provinces, and that the federal government is free to select what it wishes to investigate.

The federal government has itself already challenged Krever's right to issue the 'potential wrongdoing' notices. In this it was joined by provincial governments, private organizations and individuals. But Quebec's latest move is much more far-reaching than such earlier efforts to muzzle the inquiry, as it could limit the federal government's broad involvement in setting standards in areas such as health care.

The executive director of the Quebec branch of the Canadian Hemophilia Association, reacted to the province's claim about the unconstitutionality of Krever's action by saying those infected by tainted blood are beginning to despair, as they are coming to believe that governments do not want to know the truth about the blood tragedy. **David Spurgeon**

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Chambon: shortage of fellowships worldwide.

Panel softens cancer gene test warning

Washington. A top-level advisory panel on women's health issues to the US National Institutes of Health (NIH) last week added its voice to demands that pre-symptomatic genetic testing for breast and ovarian cancers should be carried out only within cautious guidelines.

But the Advisory Committee on Research on Women's Health (ACRWH) substantially softened the wording initially proposed by some of its members. While these had sought a virtual ban on such genetic testing outside research settings, their critics argued that such a ban would be patronizing to women.

The strength of feeling expressed in this original goal reflected deep concern over, for example, the news that the private Genetics & IVF Institute in Fairfax, Virginia, is offering \$295 screening tests for a mutation in the *BRCA1* gene, thought to confer an 80–90 per cent lifetime risk of breast cancer and a 40–50 per cent lifetime risk of ovarian cancer in Ashkenazi Jewish women with a strong family history of these cancers (see *Nature* **380**, 376; 1996).

The final ACRWH resolution urges that testing "only be conducted when accompanied by pre- and post-testing counselling which addresses the current status of knowledge", including uncertainties about how to apply genetic test results to the prevention and treatment of breast and ovarian cancers.

The resolution does not call for a ban on testing in non-research, for-profit settings. Nor is it legally binding. But as a public statement by a high-profile NIH advisory panel, it is bound to carry weight in an escalating debate. At issue is the wisdom of making genetic tests widely available in the absence of definitive data about their implications, including a test's predictive significance, and whether — and how — to proceed with medical management when women test positive.

The original resolution demanded that pre-symptomatic genetic testing for these cancers "be conducted only under hypothesis-driven, institutional review board approved research studies" which should address questions such as testing accuracy, the risks of diseases related to various mutations, and the appropriate medical management of those carrying mutations.

The final resolution also endorses research aimed at answering these questions under a new initiative by the National Cancer Institute for a National Cancer Genetics Network to provide and collect information on testing issues, and to develop a national protocol for "hypothesis-driven studies".

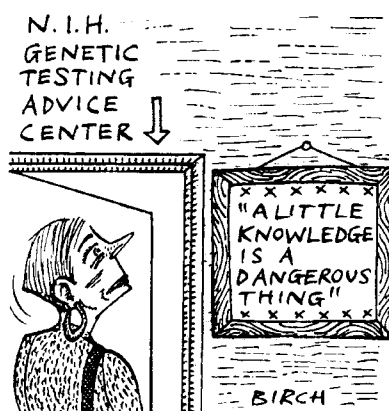
The initial resolution was developed by a subcommittee led by David Brown, professor of paediatrics, laboratory medicine and pathology at the University of Minnesota School of Medicine. On the first day of last

week's meeting, Brown said that the resolution should bar the "unlimited availability" of testing and put it "within the framework of research". But his argument that it was too soon to allow the general availability of genetic screening for breast and ovarian cancer immediately drew criticism from some female members of the committee. Over the next 24 hours the resolution was modified several times. Its final form contains no reference to limiting testing to research settings.

"The way it was changed was much better," said Linda Burhansstipanov, a committee member and director of the Native American Cancer Research Program at the AMC Cancer Research Center in Denver, Colorado. Burhansstipanov and others say that the improvement resulted from the removal of what she describes as "paternalistic language" that patronized women. Another committee member said it signalled a retreat to "the age of 'doctor knows best'".

But others — most notably, Francis Collins, the director of the National Center for Human Genome Research (NCHGR), who did not attend the meeting — disagree that the final wording is an improvement. "It does seem to open the door to carrying out testing outside of [the research] environment," said Collins.

"It's just slightly disappointing that they



chose to back away from a stronger statement," says Collins, who wrote in the *New England Journal of Medicine* earlier this year that "the technical ability to perform tests for mutations should not be confused with a mandate to offer them".

In contrast to the advisory panel's resolution, Collins says that the Hereditary Susceptibility Working Group — which he co-chairs — of the National Action Plan on Breast Cancer will publish a statement in *Journal of Clinical Oncology* next month concluding that "testing at the present time should only be carried out under the auspices of a research protocol approved by an institutional review board".

Numerous scientific groups have recently

concurred, arguing that testing for *BRCA1*, a gene which can carry mutations responsible for inherited breast cancer, should be confined to research settings. These include the American Society of Human Genetics, the National Breast Cancer Coalition and the Advisory Council of the National Center for Human Genome Research.

Furthermore, Neil Holtzman, who chairs a task force on genetic testing of the NIH/Department of Energy Working Group on Ethical, Legal, and Social Implications of Human Genome Research, says that in his opinion the *BRCA1* test does not meet the criteria, contained in a set of 'interim principles' released by the task force last month, for making genetic tests publicly available.

But refusing to endorse the non-research use of breast cancer screening would provoke "lay outrage", warned Marjorie Shultz, a professor at the University of California at Berkeley's Boalt Hall School of Law. "Can you imagine yourself saying to a woman who comes to a centre to do testing 'No you can't, unless you're a research subject?'" she asked Brown. "Yes," he immediately replied, arguing that to allow testing on demand would be like permitting patients "to go to their doctor and say 'I decide that I want dicloxacillin today'".

Others fear that a stampede of women to private genetic testing facilities such as the Virginia laboratory would deprive researchers of the subjects needed to answer critical questions. "If everyone goes to a private company and has testing, we're going to lose that data," said Kathy Hudson, assistant director for policy at the NCHGR.

In the end, the NIH is likely to have little authority to regulate private testing facilities. Instead, some experts say, this will be the responsibility of the Food and Drug Administration (FDA) under the Medical Devices Act. The FDA is expected to acknowledge its authority to regulate laboratories that market genetic test services — as well as its lack of resources to do the job — in an opinion shortly to be submitted to Holtzman's task force.

One member of the ACRWH committee argued that FDA regulation would quickly put an end to commercial genetic testing for *BRCA1* and *BRCA2* genes. "The data out there would be laughed at by most of the people in the FDA," said Edward Brandt, Jr, director of the Center for Health Policy Research and Development at the University of Oklahoma Health Sciences Center.

The resolution finally approved by the ACRWH advisory committee last week calls on the NIH to take a lead in publicizing the current lack of knowledge about how genetic test results might be used to prevent and treat breast and ovarian cancers.

Meredith Wadman

Meeting seeks to rally support for research projects

Vienna and Moscow. The United States hopes to obtain wider support at next week's G-7 meeting on nuclear safety in Moscow (see right) for a plan to provide \$3.3 million to set up a Chernobyl nuclear research centre to study nuclear safety and related issues.

Last month, the US Department of Energy and the Ukrainian ministry of environment signed a joint statement of intent to implement the programme. Both sides hope for additional investment from other countries.

Research is intended to focus primarily on how to increase the safety of nuclear reactors, how to implement appropriate decontamination programmes, and the development of systems of emergency planning in the event of any future nuclear accident.

The G-7 'nuclear summit' will also be asked to support a number of programmes discussed earlier this month by participants at an international seminar on methods of detecting nuclear and other explosive materials. These include programmes on securing the safety and preservation of nuclear and radioactive materials, preventing nuclear terrorism and cleaning up territories polluted by explosives.

The first international seminar on nuclear-physical methods of detection of hidden explosives and fissionable materials was held earlier this month in Obninsk, a town near Moscow where the first Soviet nuclear reactor was built. It was organized by the Russian Ministry of Atomic Energy, the International Scientific-Technological Centre and the Argonne National Laboratory in Illinois, United States.

The meeting was addressed by Vladimir Mikhailov, Russia's minister for atomic energy, who welcomed it as "the first open meeting of the scientists and engineers belonging to the formerly strictly secret field of research". He added that there was an "urgent necessity" to prevent the danger of nuclear terrorism and smuggling.

The Russian and foreign (primarily American) participants at the seminar discussed the theoretical and practical models of devices able to detect radioactive materials. The development of such devices is seen as one way of redirecting the efforts of highly qualified researchers and engineers previously engaged in development of nuclear weapons to the solving of peaceful tasks.

Alison Abbott & Carl Levitin

Politics dampens summit on Chernobyl's anniversary

Washington. World leaders are gathering in Moscow this weekend (19-20 April) for the first-ever summit devoted to nuclear safety issues, amid low expectations of tangible results and a strong suspicion that the summit is chiefly intended to bolster the re-election chances of Boris Yeltsin, the president of Russia.

Leaders of the seven largest capitalist economies (G-7) are meeting in Moscow with Yeltsin and senior officials of other states of the former Soviet Union (FSU) to discuss the safe operation of nuclear power plants and the secure handling of nuclear weapons materials. The meeting is timed to coincide with the tenth anniversary of the nuclear accident at Chernobyl in the Ukraine.

"I won't be disappointed, because I'm not expecting much," says Frank von Hippel, a physicist and nuclear non-proliferation expert who is a former senior official in the Clinton administration. Hippel will travel to Moscow with an independent task force that intends to press the leaders for drastic action to improve nuclear safety and security.

The so-called International Expert Task Force for the summit — a group of 46 prominent environmentalists and scientists, co-chaired by Alexei Yablokov of the Center for Russian Environmental Policy in Moscow and Tom Cochran of the Natural Resources Defence Council (NRDC) in Washington DC — is calling on the FSU states to earmark a number of dangerous nuclear power stations for shutdown over the next ten years, and on G-7 to set up a \$10-billion "revolving fund" to support non-nuclear energy sources to replace them.

According to US officials, however, there is no chance of the summit agreeing on anything so ambitious. Instead, it will seek to cement the more incremental approach currently being undertaken to nuclear safety and security issues.

The communiqué issued at the end of the meeting will probably express continued support from the G-7 for training and regulation at nuclear plants in the FSU, the officials said. It may also pledge backing for a study on the economics of decommissioning some nuclear plants, and the construction of a small demonstrator plant for the disposal of plutonium, the officials said.

In addition, Russia may be persuaded to join new negotiations on how to implement an existing agreement on the exchange of classified information about the status of its nuclear weapons materials.

The G-7 countries are in no position to tell the FSU states to close reactors. Indeed

IMAGE
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Lonely legacy: contaminated vehicles at Rassohka, the largest storage point in the exclusion zone.

none have been shut since the Chernobyl accident, despite allegations that many are intrinsically unsafe. But G-7 countries hope that an outside study could persuade the states that decommissioning is the most economically viable option in some cases.

Kristen Suokko, associate director of international nuclear safety at the US Department of Energy, said that the summit would "provide an impetus" to existing efforts to improve reactor safety. These include US\$180 million spent by the United States so far on more than 170 separate projects, ranging from system upgrades to training schemes, under a programme managed by the Pacific Northwest Laboratory in Washington state.

But some environmentalists have already written off the summit as a missed opportunity. Christopher Flavin, a nuclear power analyst at the Worldwatch Institute in Washington DC, says that it will pursue "a very mixed and confused agenda, underlying a desire to prop up" the nuclear industry in the FSU "while the whole thing is meant to prop up Yeltsin" in his bid for re-election. "The results will not really merit a summit," he predicts, adding that they could have been achieved "at a much lower level".

Colin Macilwain

Indian opposition 'will

New Delhi. The conservative Bharatiya Janata Party (BJP) of India, the major opposition to the ruling Congress party, has said that if it comes to office through next month's general elections it will hasten the deployment of the indigenous intermediate-range ballistic missile Agni, as well as reviewing India's nuclear policy and increasing funding for research in defence and security related areas.

The BJP's election manifesto says the party's main goal would be to take corrective actions on the Congress government's policies which, it says, have put the country's security in peril. The Congress govern-

German minister bemoans weak response

Vienna. Angela Merkel, Germany's environment minister, last week attacked a conference on the Chernobyl accident organized by the International Atomic Energy Agency (IAEA) for failing to issue a strong statement on the closing down of the Chernobyl reactor and other similar nuclear reactors, which she argued could have been taken to next week's G-7 meeting on nuclear safety (see opposite).

Instead, the four-day technical conference, which summarized the medical and sociological consequences of the accident, as well as the measures already taken to ensure future reactor safety, drew a relatively mild set of conclusions about whether reactors of the RBMK type — such as those at Chernobyl — should be decommissioned.

A joint team of German, French and Russian experts concluded that some fundamental failures in RBMK design have been corrected in many plants, even though important safety deficits, which vary from plant to plant, remain. Moreover, it stated, the lack of understanding of western experts of detailed engineering aspects of RBMK reactors made it difficult to recommend closure of specific plants.

The compromise statement reflects the different interests of the West, which is concerned primarily about the safety of nuclear power in the newly independent states (NIS), and of the countries themselves, which are concerned about its price.

Adolf Birkhofer, head of the German Society for Reactor Safety, who advises the German government on such issues, told the meeting that the complicated design of the 15 operating RBMK reactors created so many potential problems that they should all be shut down as soon as possible. But closure can only be authorized by the states in which they are located, and Lithuania, Ukraine and Russia each maintain that they can continue to operate the reactors securely.

Western European countries now accept

back nuclear research'

ment has opposed the development of nuclear weapons, and had stopped further tests on Agni.

BJP says it is committed to a nuclear-free world. But it adds that it will continue to develop nuclear weapons if countries that now have atomic bombs do not renounce them. It is opposed to the comprehensive test-ban treaty in its present form, and "will not agree to the fissile material control regime". The party, which currently rules three states, is putting up candidates in all of India's 22 states, and hopes to obtain a majority in the 520-seat lower house of the parliament.

K. S. Jayaraman

that their wish for serious discussion about closure of the 15 plants will not take place at next week's G-7 meeting, and are concentrating their efforts of the complete closure of Chernobyl, where two reactors continue to operate.

Last December, the G-7 countries signed a memorandum of understanding in which they pledged ECU2 billion (US\$2.5 billion) to help support the closure of the remaining two reactors, the completion of alternative

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Human cost? Children in the leukaemia ward for the victims of Chernobyl, at Kiev children's hospital.

electricity generating plants, and strengthening of the so-called sarcophagus, the containment structure that was built over the destroyed reactor, which is feared to be unstable.

Since then, behind-the-scenes negotiations have raised this figure to ECU3.1 billion, with Ukrainian environment minister Yuri Kostenko holding out for more. Kostenko is also negotiating over how much

of the sum now on the table would be in the form of loans, and how much in straight aid.

Even if an agreement over Chernobyl is reached, it is unlikely to come into force until 2000 at the earliest, and it would be several more years before the reactors were actually switched off. Total decontamination of the area around Chernobyl is estimated to require at least 15 years to complete.

Merkel, who chaired the IAEA conference, stressed the importance of the closures if public faith in nuclear power is to be restored. "If we want to [justify] use of nuclear energy, the safety of nuclear power plants has to be given absolute priority," she said.

The conference further concluded that the medical consequences of the Chernobyl accident were quite different from original predictions. An unexpectedly high increase in thyroid cancers in young children has been reported. But there is as yet no evidence of an increase in leukaemias among so-called 'liquidators', those who were exposed to high levels of radiation when working in the heavily contaminated zones after the accident.

Similarly there is as yet no firm scientific evidence for an increase in other solid tumours that can be ascribed to the accident. But stress — a consequence of the prevailing fear that the health, and therefore future, of individuals have been irreversibly harmed — was generally acknowledged to be one of the most debilitating consequences of the accident.

Alison Abbott

Michael J. O'Brien/Panos Pictures

African continent goes 'nuclear-free'

Cape Town. African countries last week signed a treaty declaring a desire to keep the continent free of nuclear weapons. At a ceremony in Cairo, 50 of Africa's 53 nations signed the Treaty of Pelindaba, banning the possession, testing or storing of nuclear arms.

Ironically, the treaty is named after the site occupied by the South African Atomic Energy Corporation near Pretoria, where it was concluded. This is where the only known weapons-grade uranium was produced on the continent, used in the late 1980s to manufacture South Africa's six nuclear devices, which have subsequently been dismantled.

The treaty, which seeks to promote the use of nuclear science for peaceful processes, creates the world's third nuclear-free zone, following similar pacts by Latin American and South Pacific countries. Madagascar, Somalia and the Seychelles did not sign the treaty for "political or technical" reasons, accord-

ing to the Egyptian foreign ministry.

The United States, France, Britain and China were also present to sign an annex to the pact promising not to use or threaten to use nuclear weapons against any African country. The fifth nuclear power, Russia, balked at signing, citing the presence of a US military base on the Indian Ocean island of Diego Garcia, considered African territory under the treaty.

The treaty will be monitored by the African Commission on Nuclear Energy (Afcene), which is expected to employ a small permanent staff whose work will be overseen by 12 commissioners. South Africa has offered to host Afcene, and this offer has been taken up by the Organisation of African Unity.

In addition to South Africa, a key role was played in the negotiations by the government of Egypt, which already faces a possible nuclear threat in the Middle East from Israel. **Michael Cherry**

UK position in particle physics 'threatened by business plan'

London. Britain's particle physicists are complaining that the latest business plan produced by the Particle Physics and Astronomy Research Council (PPARC) for 1996 would, if implemented, impose significant damage on the United Kingdom's ability to remain at the forefront of research in particle physics.

Objections to PPARC's plan centre around its proposal to reduce the UK's contribution to the European Laboratory for Particle Physics (CERN), while setting an overall ceiling for particle physics expenditure that would include the UK domestic programme. If CERN refused to accept the reduced subscription, cuts would have to be made in the domestic programme.

Peter Dornan, professor of physics at Imperial College, London, says that PPARC policy "would make any kind of long-term planning totally impossible, and limit what we can do and what we are committed to do". He adds: "It would have disastrous consequences."

Ken Pounds, chief executive officer of PPARC, says that he shares physicists' concerns at the increasingly inadequate funding of particle physics in Britain, but points out that this is partly due to the steep rise in the CERN subscription, and that the initial cuts in the astronomy programme last year were even bigger. □

Monsanto set to buy Agracetus

London. W. R. Grace & Co., a major world supplier of chemicals for specialized health-care services, has announced that it plans to sell the transgenic plant business of its subsidiary Agracetus to the Monsanto Company for US\$150 million.

Grace will retain Agracetus' human gene therapy business, which will operate as Auragen Pharmaceuticals, Inc. Robert T. Fraley, pres-

ident of Monsanto's Ceregen unit, which develops new agricultural products, said that bringing together Agracetus' technical depth and Monsanto's application and commercial abilities will speed the development of new products.

During the 1980s, Agracetus pioneered technology advancement in the genetic engineering of plants, including generating the world's first transgenic varieties of cotton, soybeans and peanuts. □

Work to start on Munich reactor

Munich. The Technical University of Munich is to start the immediate construction of its controversial research reactor, known as the FRMII, in Garching, near Munich, following the granting of a partial construction licence by the Bavarian environment minister last week. The licensing, which had been delayed for six months, was the final obstacle faced by the university before getting the DM720-million project under way.

The reactor has come under heavy criticism, both locally and internationally, because it will burn weapons-grade highly enriched uranium (HEU). This is contrary to an international agreement, signed in 1978, known as Reduced Enrichment for Research and Test Reactors, which aims to eliminate commercial trade in HEU. □

India signals priority for thorium

New Delhi. Anil Kakodkar, a reactor engineer who played a key role in the design and testing of India's first nuclear explosive device in 1974, has been appointed director of the Bhabha Atomic Research Centre (BARC) in Mumbai, formerly Bombay. Kakodkar, who succeeds A. N. Prasad, was previously head of the centre's reactor design and development group. Soon after his appointment, he announced that BARC's main thrust would be to use India's thorium reserves, estimated at 360,000 tonnes, for power generation.

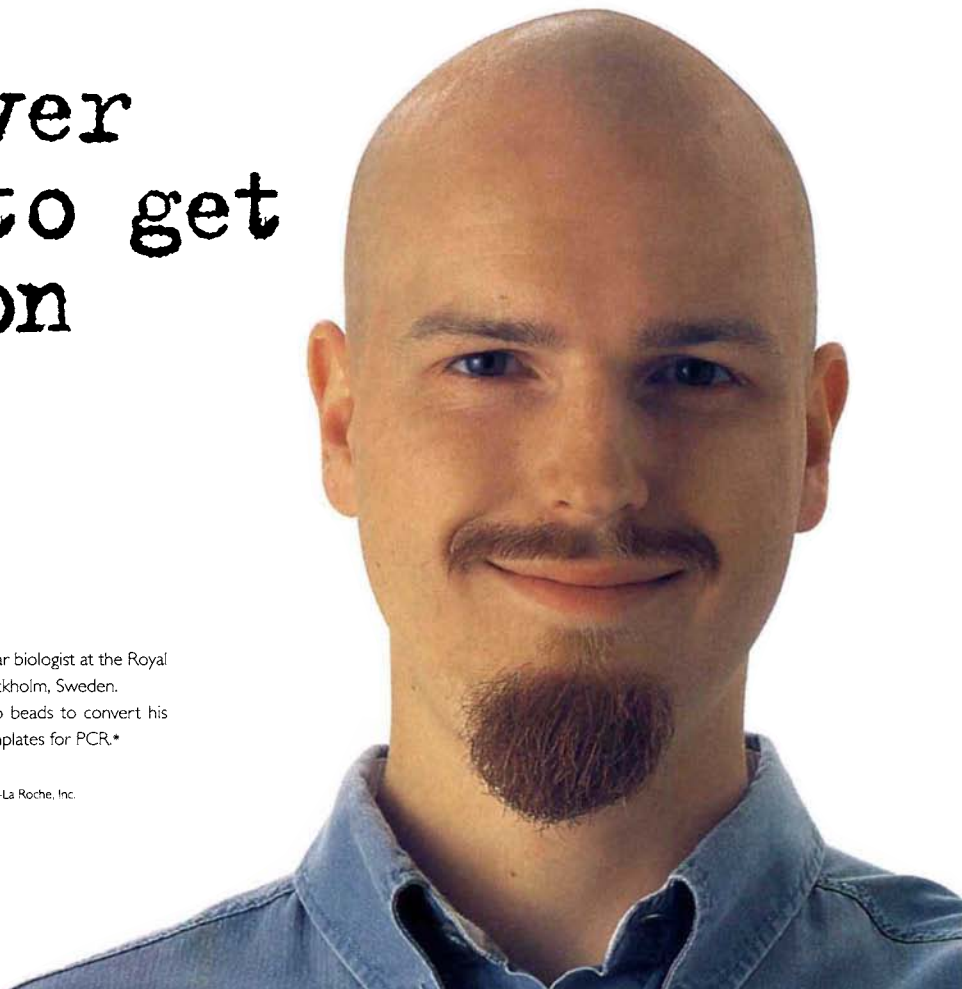
Kakodkar's appointment over the heads of several more senior

Patrik never fails to get a reaction

Patrik Samuelson is a molecular biologist at the Royal Institute of Technology in Stockholm, Sweden.

Patrik uses Ready-To-Go beads to convert his RNA samples into cDNA templates for PCR.*

* PCR is a patented process of Hoffmann-La Roche, Inc.



staff members is seen as a signal that the Indian government is keen to promote research on thorium. In the past few years, he and his team have designed a water-cooled reactor able to generate power by consuming thorium and a mixture of plutonium and uranium oxide fuel. With Kakodkar now at the head of the centre, work on this project is likely to accelerate. □

Unilever centres on Bangalore

New Delhi. The Anglo-Dutch conglomerate Unilever has chosen Bangalore as the site for its fifth global research and development centre, the first to be established outside Europe and the United States. Expected to cost about US\$30 million, the new centre will make use of the rich and relatively inexpensive scientific talent available in India to support the company's global activities.

Unilever currently operates four research and development centres at Port Sunlight and Coleworth, both in the United Kingdom, Edgewater in the United States and Vlardigen in Holland. The Indian centre is expected to be in operation by May 1997. □

Japan eases rules on DNA vectors

Tokyo. Japan's Science and Technology Agency (STA) has announced a revision of the rules on the approval of some recombinant DNA experiments, making it easier to use viral vectors and recombinant plants and animals developed overseas. Under the new guidelines, experiments using viral vectors will no longer need individual scrutiny by a committee within the STA; instead they will be able to gain approval from the safety committees of individual institutions.

Similarly, recombinant organisms produced overseas will no longer require individual assessment by the STA before they can be imported and used in experiments. Guidelines for field release of recombinant organisms have not been formulated as no applications for such experiments have yet been made, says the STA. □

US foreign policy goes greener

Washington. Warren Christopher, the US Secretary of State, last week promised that environmental issues will play a greater role in US foreign policy in the future as part of a "far-reaching agenda to integrate fully environmental objectives into our diplomacy".

In an address delivered at Stanford University in California, Christopher said this agenda includes the creation of 'environmental hubs' at more than a dozen US embassies around the world to coordinate environmental activities on a regional scale. Environmental problems are becoming increasingly important to US national interests because of their trans-border nature, said Christopher.

He also announced two new State Department initiatives — an annual report on global environmental challenges, to begin in 1997, and a "Partnership for Environment and Foreign Policy" involving US businesses, environmental organizations and other groups working together on environmental issues.

The agenda outlined by Christopher is in fact partly a business strategy. "We are committed to helping US companies expand their already commanding share of a \$400 billion market for environmental technologies," he said. Although the speech focused mostly on existing programmes, US environmental leaders said it was significant for its high-level acknowledgement of what had previously been non-urgent issues. □

Prize for biotech entrepreneurs

London. Britain's Biotechnology and Biological Sciences Research Council has launched a 'new entrepreneurs' prize to encourage post-graduate researchers to consider industrial applications of their research. Eight teams will compete to produce the best business plan for a start-up company based on their research. Industrial and commercial tutors will be on hand to give advice. □

Do you always get a reaction when converting RNA into single-stranded cDNA templates for RT-PCR? Patrik does—thanks to a revolutionary polymeric bead that makes these critical conversions for him in new Ready-To-Go™ Kits.

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Advanced Technology Program

SIR — Your leading article "Surrender on technology support" (*Nature* 380, 185; 1996) accurately points out that, in a time of tight budget constraints, there is no place for the Advanced Technology Program (ATP) in the US federal budget.

What is erroneous, however, is the characterization of comments I have made about the link between the awarding of ATP grants and political contributions, where you describe my claim that grants are being used as political patronage as "an unsubstantiated slur" on staff at the National Institute of Standards and Technology (NIST). A policy analysis entitled *Ending Corporate Welfare As We Know It*, written by Stephen Moore and Dean Stansel of the Cato Institute and released in May 1995, states:

Many of the top recipients of technology research grants awarded by the Clinton administration were also substantial contributors to the Clinton campaign or the Democratic National Committee. For example, Table 1 [on page 8 of the study] lists eight Fortune 500 firms that were multi-million-dollar award winners of the Advanced Technology Program or the Technology Reinvestment Project in 1994 that were also large Democratic campaign contributors, according to Federal Election Commission (FEC) data compiled by Common Cause. At the very least, these golden handshake programs create an impression that government is for sale.

NIST is a vital part of the federal science infrastructure of the United States. The scientists carrying out basic research at the NIST laboratories are topnotch and should be commended for their work. The real slur on these employees is the Clinton administration's budget request for fiscal year 1997. While the administration continues to request money for ATP, the president's request for the scientific and technical research and services, known as the NIST "core", was lower this year than last.

Robert S. Walker
(Chairman)

*Committee on Science,
US House of Representatives,
Washington DC 20515, USA*

SIR — You characterize the US Commerce Department's Advanced Technology Program (ATP) as "an expensive act of faith". Without wishing to get into a theological debate, there are some significant leaps of faith — well, perhaps "leaps of doubt" — in your argument.

You say "no-one will ever know" if the ATP achieves its objective of significantly enhancing the US economy by enabling industry to develop economically important but high-risk technologies. In fact, we will

almost certainly have a much better understanding of the actual impact of the ATP on industry and the US economy than for virtually any other comparable programme in either the public or private sectors.

We agree that government programmes, particularly funding programmes like the ATP, should be monitored carefully and evaluated. The ATP has emphasized rigorous measurement and evaluation of its procedures from the start. These range from periodic surveys and a sophisticated business reporting system to track the progress of individual projects to detailed case studies and econometric analyses to help project impacts from individual companies to an industry or the economy as a whole. In addition to its own in-house staff of business and economic experts, the ATP regularly consults leading academic economists to develop state-of-the-art methodologies for measuring programme impact.

Indeed, while it is clearly premature to talk about the long-term effects of the ATP, short-term effects have been well documented. Companies have committed approximately \$1 billion of their own funds to research projects that by their own analyses would not have been attempted, or would have been pursued at a significantly lower level of effort, without the ATP. Research and development schedules have been accelerated significantly. Early ATP projects are already resulting in business growth because of the availability of new technologies. More companies are establishing mutually beneficial research and development alliances with customers, suppliers and even competitors as a result of the ATP. All of these trends augur well for the programme's long-range impact.

You argue that, as an experiment, the ATP has no "accepted" design, no end-points, and no control. True, we can't run a control version of the United States without the ATP. By that facetious standard, virtually no government programme — including support for basic research — and precious few private enterprises would ever be pursued.

You feel that it will be difficult to justify the Clinton administration's insistence on the ATP "at a time when the US government has no money for the excellent university science funded by the National Science Foundation". In 1995, the federal bill for research and development at universities and colleges was \$13 billion. The ATP budget was \$341 million. Let's keep both a sense of proportion and the ATP.

Mary L. Good

*(Under Secretary for Technology)
US Department of Commerce,
Washington, DC 20230, USA*

Impact factors of Russian journals

SIR — According to the *Journal Citation Reports (JCR)*¹, the impact factor of journal X is defined by the following rule: "The JCR impact factor is basically a ratio between citation and citable items published. Thus, the 1988 impact factor of journal X would be calculated by dividing the number of all the SCI, SSCI and A&HSCI source journals' 1988 citations of articles journal X published in 1986 and 1987 by the total number of source items it published in 1986 and 1987"¹.

We should like to add to the discussion in Correspondence^{2,3} by pointing out the difficulties of defining the impact factors (IFs) of Russian journals. First, the list of citing/cited journals in *JCR*, as with our such indices, is not complete. There is clearly uncertainty in impact factors that is difficult to quantify. Second, inaccuracy arises from the timelag before publication of a journal. The lag can be defined as the time between submission and publication.

Consider the typical publication lag in Russia in the previous decade. In the 1980s, the IFs of Russian journals were relatively high and these may have been the most favourable years for Russian science.

In the late 1980s, publishing an article in a Russian journal became very complicated, with discussion at different seminars and commissions, mailing to the editor, printing and mailing to the libraries. Sometimes a paper has to be returned for revision. So a timelag of 1.5–2 years may not be unusual. The IFs of Russian journals at best are two to four times smaller because of the timelag.

Where Russian journals are translated, and on the assumption that the dates on the covers of the Russian English versions are the same, the timelag for citation of translated Russian journals may be much greater than that of those originally published in English.

At the beginning of the 1980s, translation and publication in English took up to two years. This decreases the IF of the journal by a factor of four or more immediately. The same conditions do not apply now, but it seems nevertheless to be the case that the impact factors of Russian journals are undervalued.

Nikolaj I. Sorokin

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1. *Journal Citation Reports* p.10A (1989).

2. *Nature* 374, 492 (1995).

3. *Nature* 376, 720 (1995).

An underground route for the water cycle

Thomas M. Church

Water flows from the land to the sea in rivers — that is the classroom picture that seems too self-evident to question. But there is evidence that a comparable amount may flow underground directly into coastal waters.

WHAT supplies salt and sediment to the oceans? The traditional answer is weathered rock material borne primarily by rivers. During past decades, however, other sources have been discovered or re-evaluated in order to explain the chemical balance of sea water. One discovery is the deep submarine source from the weathering of fresh basalts by hydrothermal recirculation along mid-ocean ridges. Another source being re-evaluated is the atmospheric deposition onto the ocean of dust and trace elements, some of which are essential micronutrients for plankton. A third, which has been suspected as a source for some time, is the direct flow of water into the sea through porous rocks and sediments, called submarine groundwater discharge (SGWD), but quantitative evidence has been lacking. Now Moore on page 612 of this issue¹ reports that, over several hundred kilometres of coastline in the southeastern United States, SGWD is comparable to the observed discharge from rivers.

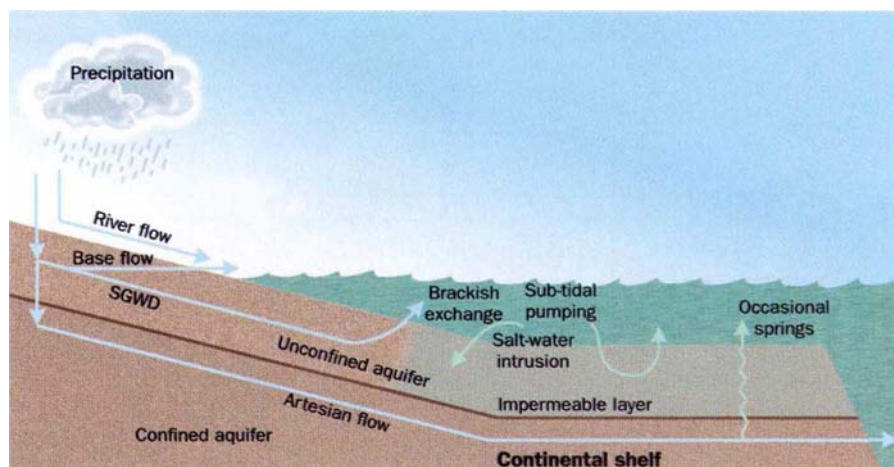
The conclusion that SGWD may be a major geochemical supply to the ocean should come as no surprise when one considers that 97% of the Earth's liquid freshwater reservoir is groundwater. The 'base flow' of rivers originates largely as groundwater seepage from aquifers along their banks, and in arid areas can continue to flow beneath seasonally dry river beds. So river inputs already contain some fraction of water that has passed first into aquifers during the weathering process. Furthermore, SGWD is believed to supply nutrients to vegetation in coastal lagoons^{2,3}, and there are many observations of aquifer discharge to the coastal ocean⁴. Perhaps the most obvious examples are in porous limestone terrains such as the Florida platform and the areas bordering the Mediterranean and Red Seas and the Persian Gulf. There, the open structures of the weathered limestone carbonates host submarine rivers with upwelling plumes offshore where ancient mariners were able to restock their freshwater supplies.

Previous estimates of the amount of SGWD worldwide range from 0.01 to 10% of surface-water runoff. A better quantification has been difficult because there is not enough information on hydraulic pressure gradients and transmission coefficients along the world's coasts. From

measurements in confined coastal areas and inland seas, SGWD can amount to 3% of precipitation or 10% of stream flow⁵. It seeps from unconfined aquifers into the near shore⁶ or is discharged from confined (artesian) freshwater aquifers found beneath continental shelves further from shore⁷.

A more fruitful approach to quantifying SGWD is to use tracers that are naturally enriched in ground water. One such set of

change of brackish water between fresh and saline aquifers in the coastal zone¹⁰. One mechanism is the pumping produced by the rise and fall of the tides. (It has been calculated that subtidal pumping takes only about 14,000 years to 'sand filter' the volume of the ocean¹¹.) However, there must always be a net discharge from the land to the ocean driven by the upland pressure of terrestrial aquifers. Moore estimates that submarine fluxes of



The routes of water flow into the sea. Radium leached out of the rocks and sediments can be detected in the sea, and betrays the magnitude of the underground flow. Off the Carolina coast of the United States it is about 40% of that in rivers, far greater than had been thought and perhaps enough to change our ideas about the chemical balance of the oceans.

tracers is radium (^{226}Ra) and its daughter, radon (^{222}Rn), both members of the uranium (^{238}U) decay series. Coastal excesses of these nuclides have been observed in offshore hot springs⁸ and in seepage onto continental shelves⁹. Unlike radon, whose half-life is just a few days, radium has a sufficiently long half-life (1,620 years) to build up during uranium decay and become enriched, particularly in calcareous rocks naturally rich in uranium.

Moore measured the distribution of ^{226}Ra in near-shore shelf-waters of the South Atlantic Bight. He estimates that all river sources, including the desorption of radium from the fresh river sediments within estuaries, can account for no more than half of the near-shore radium. There must be about 40% as much submarine groundwater discharge as there is river flow, a surprisingly large percentage compared with other coastal estimates.

To maintain a large radium discharge requires an effective hydrological ex-

at least 5 litres per square metre per day over the inner shelf are needed to account for the radium excesses in the South Atlantic Bight, consistent with localized measurements⁶.

It takes hundreds of years for radium levels to be regenerated radioactively in sediments that have been depleted by saline exchange, so the observed excess of radium from SGWD requires a fresh modern source. Moore hypothesizes that salt water has recently intruded into previously uncontaminated freshwater aquifers along the coast. It is probable that in modern times increasing demands for fresh water have lowered coastal aquifers, and dredging of shipping channels has breached them. The aquifers then lose their radium either as they drain or during tidal pumping.

Long-term relief of salt-water intrusion into coastal aquifers can come from water conservation, from cessation of dredging, or eventually from a drop in sea level,

which has undoubtedly occurred repeatedly during the glacial-interglacial cycles of the past million years. During glacial times, fresh water would have recharged coastal aquifers and a greater hydrostatic head would have increased groundwater inputs to the coastal seas. That could have produced an increased flux of groundwater chemicals to the open ocean during glacial times, many of which are used as indicators of past ocean change.

The conclusion that large quantities of SGWD are entering the coastal ocean has the potential to radically alter our understanding of oceanic chemical mass balance. It could rival hydrothermal inputs in magnitude, and indeed there is coastal hydrothermal recirculation in raised carbonate platforms such as Florida¹². The significance for chemical input to coastal ecosystems could be equally important.

Potentially, SGWD and the reverse contamination of coastal aquifers by sea water constitute a double impact on the coastal zone. As sea level rises, as demands for coastal freshwater increase, and as navigational dredging is main-

tained to even greater depths, the interaction between saline and freshwater aquifers is likely to increase and become more widespread. This has serious implications for the conservation of potable groundwater in the coastal zone, and the prudent management of coastal engineering activities. □

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ASTRONOMY

The formation of filaments

Peter Coles

ONE of the most important and difficult tasks facing modern cosmology is to explain the complex pattern of galaxy clustering revealed by redshift surveys. Prominent among the features one's eye identifies in these maps of the nearby Universe are long chains of galaxies, usually known as filaments, often stretching for tens of megaparsecs. Numerical simulations of the development of clustering have been able to reproduce features qualitatively similar to those observed, but a full theoretical understanding of their origin has hitherto been lacking. Bond, Kofman and Pogosyan, reporting on page 603 of this issue¹, have sought to remedy this shortcoming. Using a simplified dynamical model for the evolution of structure, they claim that the present-day distribution of filaments can be traced back to spatial correlations present in the primordial fluctuation field before much dynamical evolution took place.

Gravitational instability is the mechanism by which most theories of structure formation are able to produce large-scale density inhomogeneities, such as clusters and superclusters of galaxies, without violating the constraints imposed by the uniformity of the cosmic microwave background². Its theoretical essence was first elucidated by Sir James Jeans³ — small initial perturbations in the density of a largely homogeneous and isotropic Universe become amplified by the progressive

action of gravity, as regions with higher than average density gradually accrete material from their surroundings.

But although the physical mechanism is simple, there are a number of obstacles to the construction of a complete theory of cosmological structure formation based on it. In particular, the action of the gravitational instability is affected by the form and quantity of the matter the Universe contains. Fashionable theories invoke a dominant component of non-baryonic matter (unlike familiar matter based on protons and neutrons) which can be one of two generic types. Fast moving or 'hot' dark matter particles (HDM) erase structure on small scales by free-streaming effects; slow moving or 'cold' dark matter (CDM) first forms small-scale clumps which then merge to form more massive objects. These two alternatives thus furnish two different scenarios for structure formation: the 'top-down' picture exemplified by HDM first produces superclusters, which subsequently fragment to form galaxies, whereas CDM produces a 'bottom-up' model in which galaxies form first and then cluster together.

The complexities of clustering evolution in the nonlinear regime (when spatial variations in the density are comparable to, or larger than, the mean density) are such that analytical progress is difficult. Theorists often resort to the brute-force approach of numerical simu-

lations which explicitly calculate the forces between millions of particles. But to simulate is not necessarily to understand, so mathematical techniques are needed that are capable of capturing the essential characteristics of the clustering pattern without solving the full dynamical problem⁴. In the case of top-down theories, substantial progress can be made using a simplified kinematic approach known to the pundits as the Zel'dovich approximation⁵. Bottom-up hierarchical clustering, however, involves more complicated small-scale interactions and has proved a much tougher nut to crack. Until recently, the only analytical techniques available for this problem were statistical rather than dynamical (see ref. 6, for example).

The approach adopted by Bond and colleagues¹ is founded upon two important insights. The first is that, even in hierarchical theories, the evolution of large-scale structure can be accurately described by the simplified dynamics encapsulated in the Zel'dovich approximation⁷. One can therefore separate out the effect of long-wavelength fluctuations, which evolve smoothly, from that of small-scale modes, which are much more chaotic. The appropriate scale for this separation to be performed is not obvious, but Bond *et al.* suggest that it corresponds to wavelengths of the order of tens of megaparsecs, roughly the scale traced by the distribution of galaxy clusters. The second essential insight is that small-scale structure (the difficult, nonlinear bit of the problem) does not lose its memory of properties intrinsic to past fluctuations, when evolution was still linear.

Using these ideas to build on an older and very influential idea, that clustering is, at least partly, determined by spatial correlations between peaks in the initial density field⁸, Bond and Myers⁹ have produced a formidable analytical machinery that accounts more carefully for the effect of large-scale dynamics and small-scale collapse on evolving structures by incorporating the so-called 'peak-patch picture'. This comprises a nested hierarchy of primordial density peaks described by, among other things, their initial density and the tidal strain induced on them by the surrounding matter. Spatial correlations between two or more nonlinear structures can be traced back to correlations present in the density and gravitational tidal fields at the initial locations of the peaks that end up in such structures.

Bond *et al.* emphasize the importance of tidal effects in shaping filaments as part of a 'cosmic web' of connecting peaks. Their argument is that there are strong statistical correlations in the orientation of the initial tidal distortions of neighbouring proto-clusters due to the relatively large coherence length of the tidal field, and that these correlations are pre-

served as the density field evolves. As clustering proceeds into the nonlinear regime, the peak patches making up clusters first collapse, then matter is channelled into the correlation 'bridges' linking them, so forming the prominent filaments.

This view of goings-on is somewhat contrary to the accepted wisdom of how large-scale structure forms, which has been greatly influenced by the legacy of Zel'dovich. In pure Zel'dovich dynamics⁷, the first structures to form are not clusters or filaments but two-dimensional objects known as 'pancakes'. Neighbouring pancakes intersect in a one-dimensional structure, and it therefore appears natural to interpret filaments as formed by material draining into such a structure from the pancakes defining it. Material would then be expected to flow along filaments to the points where they intersect each other, thus forming clusters. This Zel'dovich picture is probably close to the truth in the setting for which the eponymous approximation was originally devised — when the initial density fluctuation field is smooth on small scales. But, although the large-scale density field is well-described by the Zel'dovich approximation in hierarchical models, classical Zel'dovich pancakes are probably not relevant to the problem.

Of course, many important questions remain unanswered. For example, is this theory truly dynamical, that is can it be used to trace the formation of individual structures? Or is it still essentially statistical, like its predecessors? And how accurately do the spatial positions and internal densities of filaments predicted in this theory agree with the results of full numerical calculations? It would be unwise to accept the cosmic web theory as the definitive explanation for the formation of filaments until it has been tested much more rigorously. But such a bold attempt to understand what many others have been content merely to simulate is praiseworthy in itself and, who knows, it may even persuade a generation of cosmologists to turn off their supercomputers for a while and try thinking instead. □

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Taking stock of PI-PLC

Robin Irvine

THE heart of an old adversary, an enzyme of the phosphoinositidase C family which has fascinated but baffled scientists over the years, is exposed in a paper that appears on page 595 of this issue by Essen *et al.*¹. Here at last we have a detailed structure (at a resolution of 2.4 Å) of the functional core of one member of this family, PLC-δ1. As we would hope, the structure reveals a great deal and answers several questions, yet it whets the appetite

called X and Y (Fig. 2) and these, as deduced from their commonality and from mutagenesis studies, contain the catalytic site. They all also have a pleckstrin-homology (PH) domain near their amino-terminal ends, and a C2 domain near their carboxy-terminal ends. Members of the β-subfamily are controlled by trimeric G proteins (the α and/or βγ subunits, whose relative contribution depends on the particular PLC-β involved). The γ-

family isoforms have an additional PH domain — they also have two SH2 domains and an SH3 domain, which neatly account for their now well-known regulation by tyrosine kinases.

The members of the δ-subfamily, of which we have PLC-δ1 here as an exemplar¹, are the simplest insofar as they have only the sequences common to all PI-PLC subfamilies. It seems very likely that they evolved first, because every PI-PLC cloned so far from an organism that split from the mammalian evolutionary tree at an early point (for example, *Dictyostelium*,

yeasts, higher plants, *Chlamydomonas*) is clearly a δ-isoform.

Because of this comparative simplicity, the δ-isoforms have been the most extensively studied with regard to deletions, mutations and detailed catalytic activity. At the moment we don't know how the PLC-δ enzymes are regulated by receptors at the cell surface, or even if they are at all; it is possible that they are regulated only by calcium ions. Indeed, they

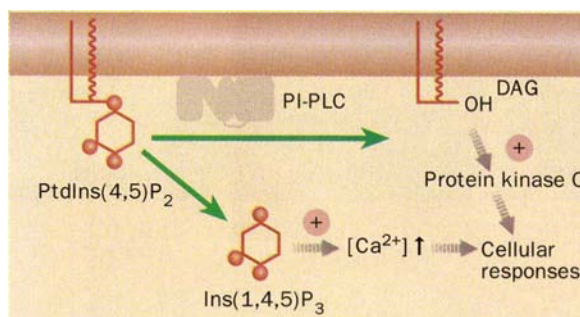


FIG. 1 Signal transduction by PI-PLCs. These enzymes catalyse the splitting in two (at the hydrophobic/hydrophilic interface) of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂), to form two second messengers: diacylglycerol (DAG) remains in the membrane and activates members of the protein kinase C family; inositol-1,4,5-trisphosphate (Ins(1,4,5)P₃) is released into the cytosol where it interacts with its intracellular receptor to mobilize calcium ions from intracellular stores.

for answers to the many more questions that are left hanging.

The phosphoinositidase C family (otherwise known as phosphoinositide-specific phospholipase C or PI-PLC) are a receptor-controlled family of enzymes that catalyse the splitting of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) in the cell membrane into its hydrophobic and hydrophilic components, namely 1,2-diacylglycerol and inositol-1,4,5-trisphosphate (InsP₃), respectively (Fig. 1). Both these compounds are well established second messengers that have had major roles assigned to them in cellular physiology. PI-PLC was actually discovered in the late 1950s, but it is only in more recent years that we have gathered together information about the whole family (for a review, see ref. 2).

There are three subfamilies of the PI-PLCs: β, γ and δ (α at the moment is lost in history), with two to four isoforms in each subfamily. All of them have two regions in common

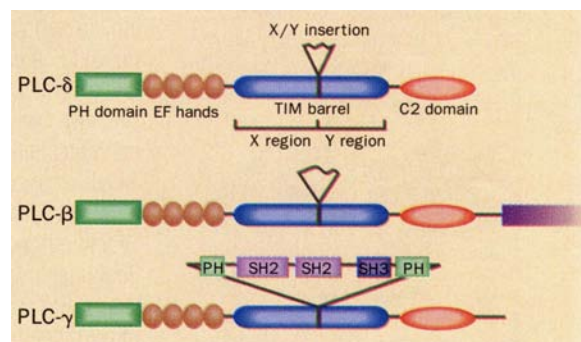


FIG. 2 Domain structure of the PI-PLC family. This summarizes the current understanding of the secondary structures of the three PLC subfamilies, emphasizing the domains common to them all (pleckstrin-homology (PH) domain, EF hands, X and Y regions, and C2), and the extra domains in the β- and γ-isoforms (carboxy-terminal region in the β-subfamily; two SH2 domains, one SH3 domain and an extra PH domain in the γ-subfamily).

may represent what was originally a Ca^{2+} -controlled diacylglycerol-generating system, which, with the advent of the Ca^{2+} -controlling function of InsP_3 and the receptor-regulated (β and γ) forms of PI-PLC, may now be part of a 'feed-forward' control of InsP_3 levels, perhaps contributing to the generation of Ca^{2+} oscillations⁵.

This latter possibility is consistent with some recent observations that are directly relevant here. PLC- $\delta 1$ has been shown to bind its product, InsP_3 , very tightly and specifically, and this binding inhibits the enzyme. This is not the case for the β and γ PLCs, and if Ca^{2+} is indeed the principal regulator of PLC- $\delta 1$ (and bearing in mind that InsP_3 increases Ca^{2+} concentrations), product inhibition would seem to be advisable in order to prevent uncontrolled amplification. The high-affinity InsP_3 -binding site has been identified as the PH domain, and the structure of this has recently been elucidated at 1.9 Å resolution⁴.

The PH domain is not essential for the catalytic activity of PLC- $\delta 1$, but its deletion does have profound effects on the enzyme's mode of action (the PH-deleted version cannot 'scoot' along its substrate⁵). Essen *et al.*¹ have, for convenience, crystallized a truncated version of PLC- $\delta 1$ that lacks its PH domain, so the structure of the functional core is revealed — it consists of EF-hand motifs, X and Y regions, and C2 domains (see their Fig. 1b), so we can now learn how the enzyme actually works.

The X region is preceded by the EF-hand domain (which was deduced earlier from a partial sequence analysis) to which it is connected by a long, structured linker. The X region is one half of what Essen *et al.*¹ call a "distorted but closed TIM barrel". The other half of the TIM barrel is the Y region. An unstructured linker joins the two — we already know from limited trypsin digestion experiments⁶ that PLC- $\delta 1$ can be split in this X–Y linker yet retain catalytic activity, because non-covalent interactions keep the two halves together. The TIM barrel is followed by a C2 domain, which has an overall structure remarkably similar to the C2A domain of synaptotagmin 1 (ref. 7). By analogy with this latter domain, and its position in the PLC- $\delta 1$ structure¹, it seems that the C2 domain serves to interact with the membrane (bear in mind that the PH domain, missing in this structure, also makes a contribution to membrane binding⁵, and the authors propose a 'tether-and-fix' contribution from the PH and C2 domains respectively¹). A hydrophobic ridge on the TIM barrel probably inserts into the membrane during catalysis (see their Fig. 5).

The catalytic TIM barrel is similar in principle to that found in a bacterial phospholipase C, and the structure of this catalytic core with $\text{Ins}(1,4,5)\text{P}_3$ bound to it

accounts in exquisite detail for many of the properties of the PI-PLCs that we have come to know and love. The extensive and specific interactions of key amino-acid residues with the phosphate groups in the inositol ring and with a calcium atom (Fig. 4c of ref. 1) can immediately explain why the enzyme prefers $\text{PtdIns}(4,5)\text{P}_2$ as a substrate; why it needs calcium ions; why it will not hydrolyse 3-phosphorylated inositol lipids or 6-derivatized PtdIns glycans; why a handful of completely conserved amino-acid residues are just that; and why the 2-hydroxyl of the inositol ring participates in the reaction.

But as we learn so much, there is so much more to learn. The EF-hand domain is without doubt crucial to the enzyme (for example, deletion of about half of it abolishes activity altogether⁶), but it is not contributing to the enzyme's Ca^{2+} requirement⁸, neither is it easy to see from the structure just what this domain is contributing, unless the C2 domain can only be held in its essential confor-

mation by a helping EF-hand domain.

The other consideration, and an enticing project for the future, is how the other domains in the β and γ PI-PLCs fold around and interact with what will surely turn out to be a core identical to that of PLC- $\delta 1$, to control the activity of these isoforms in a receptor-regulated way. But that is for another day. □

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SEMICONDUCTOR TECHNOLOGY

Quantum chaos for real

E. J. Heller

IN these times of close scrutiny of basic research funding, scientists should make a note whenever esoteric and seemingly academic concepts make their way into real devices. In this issue we have an example¹: on page 608 Wilkinson *et al.* report that 'quantum scars of periodic orbits' cause large fluctuations in current in a tunnel diode.

In a famous experiment in 1787, now often repeated in the classroom, Chladni used sand to reveal nodal patterns on vibrating square metal plates. If he had used a plate in the shape of a stadium he might have discovered scars, which are concentrations of higher wave amplitude surrounding classical periodic orbits. The fascinating association of something completely wave-mechanical (a standing wave pattern) with something completely classical (a periodic orbit bouncing off the walls of the system) is a subject within the growing field of quantum chaos.

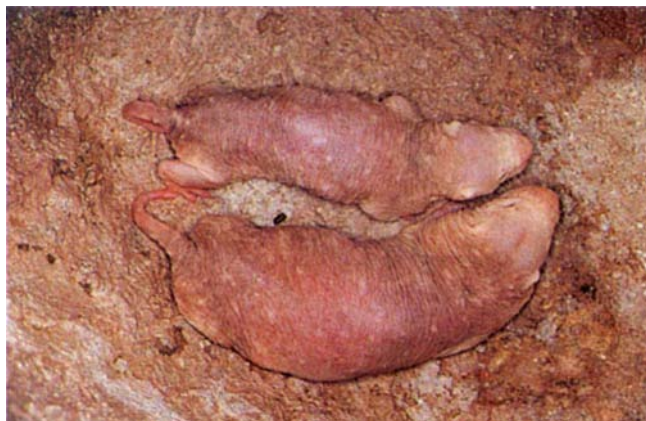
Billiards is the simplest dynamical system exhibiting full-blown classical chaos — even simple shapes like the stadium generate chaotic dynamics. Chaos is characterized by extreme sensitivity of the motion to small changes in initial conditions. Thus a tiny error in launching the 'bow tie' periodic orbit results in a ragged trajectory. Although theorists were at first a little apologetic about studying such simplified systems, experimentalists found billiards relatively easy to construct. One amazing example is a stadium billiard 'table' made from 76 individually placed

iron atoms on a surface of copper². Wilkinson and colleagues' experiment is also a system whose classical analogue is billiards. Electrons spiral around magnetic field lines and bounce elastically off the parallel walls of a very small quantum well. Because the magnetic field lines are skewed, the resulting motion is not simple and in some circumstances it is chaotic.

There is much curiosity surrounding the relationship between classically chaotic motion and quantum waves in the same system. At the beginning of the century, the old quantum theorists stumbled on classical chaos, fell down, and never really got up — the helium atom, with its two electrons, is classically chaotic, and Bohr and company never could quantize it as they had the non-chaotic, classically stable hydrogen atom. Bohr didn't know about chaos as such, but the stable periodic orbits on which they had based the quantization of hydrogen are nearly absent in helium.

Although Poincaré laid the groundwork for much of modern chaos theory through his discovery of sets of infinitely many periodic orbits in what are called homoclinic tangles, it was Einstein in 1917 who realized that chaos (which he called type II classical motion) stood in the way of what we now call semiclassical quantization. It is easy to see the attraction of this field, with an unsolved mystery going back to Bohr, Poincaré and Einstein, but the attractions go beyond the historical, because a classical or semiclassical

It's a mole-rat, Jim, but not as we know it



M. J. O'Riain & J. U. M. Jarvis

THE more monstrous of the two naked mole-rats (*Heterocephalus glaber*) in this picture is neither deficient in the product of an appetite-regulating gene, nor part of the normal range of mole-rat variation. It is a special morph adapted to dispersal, an evolutionary counter to the inbreeding depression that stalks colonial animals such as these.

The discovery of this rare dispersive morph, described by O'Riain and colleagues on page 619 of this issue, heightens the comparisons between these social mammals and insects such as termites and ants. Like social insects, mole-rat colonies are based on a small number of reproductive individuals, and society as a whole is highly organized. Again, like social insects, members of different colonies have little excuse

to meet and mingle, and when they do, the result is conflict rather than commonwealth. Inbreeding is a constant problem in all such communities, and given the customary armed truce between colonies, special mechanisms are sought to overcome it. But the last thing anyone ever expected to find was a morphological variety of mole-rat adapted to a specific task, analogous to the morphologically delineated 'castes' of social insects.

The surprise is occasioned, in part, by scarcity. Of more than 1,000 animals studied in 48 captive colonies, O'Riain and colleagues counted just 19 'dispersers'. These animals are distinctly different from the norm. Almost always male, they are fatter than average, lazy and disinclined to mate with their own kind, even the queen in oestrus — despite their having a higher-than-normal concentration of luteinizing hormone. But the dispersers show a strong tendency to leave home and mate with members of other colonies — behaviour that goes completely against the grain for the normally xenophobic mole-rats. The production of these specialized dispersers, fat-fuelled for an arduous overground mission into potentially hostile territory, is expensive of energy: only the largest and most well established colonies can afford to produce them.

But if dispersers are rare enough for their discovery to merit special comment, they are almost certainly just common enough to hinder inbreeding in the mole-rat community in general. A surprisingly small amount of gene-flow is required to maintain the heterogeneity required for reproductive compatibility between isolated populations.

Henry Gee

description of quantum states helps our understanding and may be vastly easier to calculate³ than a full first-principles quantum-mechanical calculation.

Around 1970 Martin Gutzwiller (ref. 4 and references therein) showed how to solve the old quantum theory dilemma in the presence of classical chaos. In his famous "trace formula", he showed how periodic orbits, which return repetitively upon themselves, can be used to quantize semiclassically a chaotic system to find the energy levels. The periodic orbits are always unstable and embedded in a sea of infinitely more common non-periodic orbits. The formulae he derived are quite difficult to work with, and even divergent, but others showed how to obtain more convergent expressions for the energy levels.

Despite the head start they got with Chladni's work, the wavefunctions associated with the energy levels have received much less attention than the energy levels themselves. Wavefunctions carry the information on the whereabouts of the particles in the system. Early semiclassical arguments suggested that they should be gaussian random functions of their coordinates — decidedly dull. Numerical work⁵ supported this notion. It was rather puzzling, however, that the periodic orbits should play so prominent a role in the theory of energy levels and yet have no visible effect on the wavefunctions. On the other hand, periodic orbits are exceedingly rare among the set of classical

trajectories, in fact of zero measure.

In 1984 new theoretical work revealed that some eigenstates have high probability along certain periodic orbits, providing a role for them after all⁶. The appearance of the eigenstates suggested the term 'scarred states'. A semiclassical theory for the scars uses dynamics near the periodic orbit. Scars may be viewed as quantum 'memory' of early motion near periodic orbits, a memory that is thoroughly erased classically (but not quantum mechanically) over time.

Scarring is an example of a quantum interference effect. In Wilkinson and colleagues' tunnel-diode quantum-well device, wave amplitude leaves one side of the well, spirals in the magnetic field, reflects off the opposite wall, and returns, only to repeat the motion again, essentially guided by classical periodic orbits; if the returning amplitude is in phase with the initial amplitude then a scar forms which helps to 'short circuit' the quantum well and enhance the current flow. As the voltage is changed, the energy of the electrons and their returning phase changes, causing the interference to go from constructive to destructive and back, so the scar, and thus the current, waxes and wanes.

In the past decade several types of experiment have provided direct and indirect evidence of scarred states. The most direct is seen in microwave experiments, where thin table-top cavities are built and the wavefunctions inside measured in detail⁷ (see also ref. 8 on this

and other quantum-chaos-related experiments). Indirectly, scars have been known to affect the spectra of hydrogen atoms in a magnetic field^{9–12}, and the ionization probability of hydrogen in microwave fields¹³. But the paper by Wilkinson *et al.* is especially significant, in that dramatic oscillations were caused by scarred states in a robust condensed-matter device. Interesting as the hydrogen atom and microwave experiments are, a novel tunnel diode and devices like it are more likely to show up in practical applications in the future. □

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Twitching worms catch S100

Kenneth A. Johnson and Florante A. Quiocho

SPRINGTIME finds hopeful anglers baiting hungry fish with twitching worms, both live and artificial. Fish prefer the large annelids, but Kemp and co-workers¹ have knotted on their lines the small, alluring nematode *Caenorhabditis elegans*, which twitches spasmodically when the aptly named protein twitchin goes missing from its muscle cells. And they've caught a big one!

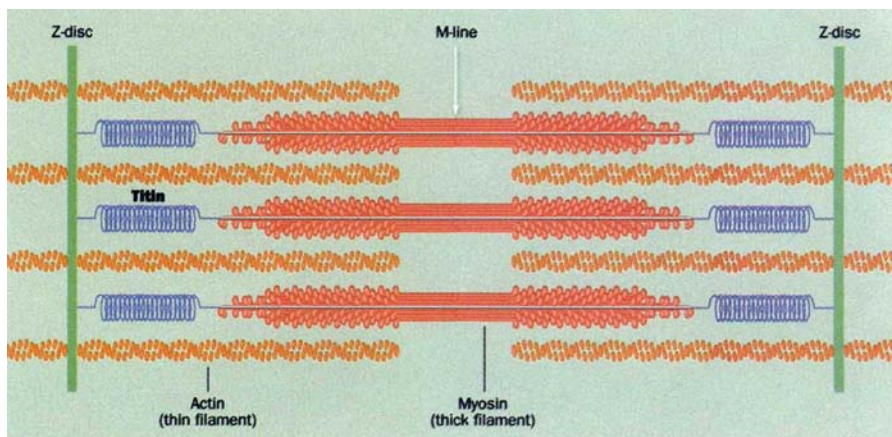
On page 636 of this issue¹, these authors report that the giant protein kinase twitchin, which has a relative molecular mass of 750K and is found in nematode muscle cells, and the protein S100A1₂, a member of the S100 family of calcium-binding proteins, make up a third new calcium-regulated system in muscle which may be of great importance in organizing muscle structure and maintaining its resting tension. They show that a fragment of twitchin containing the autoinhibited kinase domain is specifically activated in a calcium-dependent and zinc-enhanced manner by S100A1₂, but not by the S100B₂ isoform with which it shares 60 per cent homology.

Striated and smooth muscle cells of vertebrates and invertebrates contain, in addition to the filament-forming proteins actin and myosin, very large myosin-associated giant protein kinases (with relative molecular masses ranging from 700K to over 3,000K). Titin, which is found in vertebrate striated muscle, including cardiac muscle, and is one of the largest of these kinases, can stretch for 1 µm from the M-line to the Z-disc (see figure). It has been suggested that titin and titin-like proteins should be considered as a third class of muscle fibre because of their elasticity, which may help to maintain muscle resting tension^{2,3}.

Twitchin is a titin-like protein⁴. The body-wall muscles responsible for twitching in mutant twitchin-deficient nematodes were found to contain disorganized sarcomeres, which led to the suggestion that twitchin might regulate muscle contraction and be involved in the organization of muscle structure. Sequence analysis of the *unc-22* gene showed

twitchin to be a 750K protein composed of sixty-one 100-amino-acid repeats of the immunoglobulin-C2-like and the fibronectin-III-like types, with an autoinhibited protein kinase domain homologous to the well known myosin light-chain kinases (MLCK) near the carboxy terminus^{4,5}.

The twitchin kinase domain, like MLCK, specifically phosphorylates myosin light chain when it is in the form of a constitutively active fragment lacking the autoinhibitory segment⁶. In an X-ray



The sarcomere is the basic unit of muscle contraction, as explained by the sliding-filament model, in which the myosin heads of the myosin-containing thick filaments are thought to attach to the actin-containing thin filaments and to slide along them using ATP as an energy source. Two distinct calcium-dependent processes regulate contraction in smooth (involuntary, excluding cardiac) and striated (skeletal and cardiac) muscle. In smooth muscle cells, the calcium-binding protein calmodulin (in its calcium-bound form) activates myosin light-chain kinase, which in turn phosphorylates the regulatory myosin light chains located on the myosin heads of the myosin-containing thick fibres. The phosphorylated myosin heads can now grab onto the thin actin filaments and initiate muscle contraction. In striated muscle, the actin-associated complex of three troponin subunits and tropomyosin proteins prevents the myosin heads from grabbing onto the thin actin filaments in the calcium-free state. When troponin C binds calcium, the position of tropomyosin along the thin actin filaments shifts and allows the myosin heads to bind and initiate contraction.

crystallographic structure of the autoinhibited fragment of twitchin kinase⁷, the pseudosubstrate peptide segment blocked the presumed active site. Searches for an activator indicated that calmodulin, a calcium-binding protein that activates MLCK, binds in a calcium-dependent manner to an autoinhibited fragment of the twitchin kinase, but does not activate

it⁸. Titin also contains a presumed calmodulin-binding sequence⁹.

Calmodulin and S100A1₂ could be opposing effectors in this new system, just as they are in their effect on the activity of glycogen phosphorylase *a* (calmodulin indirectly activates by stimulating glycogen phosphorylase kinase; S100A1 inhibits the enzyme directly), and in the calcium-induced release of calcium ions from the sarcoplasmic reticulum in skeletal muscle (S100A1 stimulates; calmodulin inhibits).

The first two proteins of the S100 family, S100A1 and S100B, were discovered thirty years ago in a subcellular fraction of bovine brain that contained proteins soluble in 100 per cent (~4 M) ammonium

sulphate at pH 7 (ref. 10). The sixteen or so members of the S100 family have relative molecular masses near 10K and regulate many different target proteins: of special interest is the role of both S100A1 and S100B in neurite extension of cultured neuronal cells; also, S100B may contribute to the progression of AIDS and Alzheimer's disease. It has been found that S100A1 and S100B are upregulated in some cancer cell lines, and their abundance appears to be inversely related to a good prognosis.

By sequence homology, the S100 proteins are believed to have two helix-loop-helix calcium-binding regions, called EF-hand domains, connected by a short linker. An NMR structure of calcium-free S100A6 (ref. 11), which has been implicated in the regulation of cell growth, revealed an antiparallel homodimeric fold that is unique among the

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calcium-binding proteins and which is probably shared by the rest of the S100 family. It has been suggested that non-covalently associated dimers of S100 proteins are the functional form inside the cell¹⁰, whereas intercellular forms may function as dimers covalently linked by disulphide bonds. A wide cleft formed upon dimerization may be the binding surface for target proteins, which could be modified further by calcium binding to one or both of the EF hands¹¹. This would be analogous to the changes that occur on the binding surfaces of calmodulin as it goes from the calcium-free state to the calcium-saturated form¹²⁻¹⁴.

The kinase function of twitchin may be temporally distant from the function of the many fibronectin-III-like and immunoglobulin C-like repeats and could be controlled by the expression pattern of S100A1₂. It may be that the kinase activities of twitchin and titin are more important developmentally in muscle formation

and organization than they are in established muscle function. The twitchin phenotype in the nematode *C. elegans* can be rescued by missense mutations involving the myosin head region, suggesting that twitchin interacts with the myosin heads, presumably through its kinase domain. However, twitchin's role in phosphorylating the regulatory light chains of myosin is not obvious because molluscan muscle contraction is thought to be regulated by the direct binding of calcium to myosin heads.

The disparate functions of the giant kinases are more readily examined in the fruitfly *Drosophila melanogaster*, which contains in its muscle cells a titin-like protein, projectin, which has similar structure and functional domains¹⁵. Two projectin mutants were lethal at prehatching stages of development, suggesting that the mutant fly embryos lacked the necessary muscle strength for hatching¹⁶. Projectin appeared to help regulate the length of

myosin filaments *in vitro*¹⁷. P-element insertion of mutant genes into fly eggs can replace normal genes and thus tease apart the two proposed functions of organizing muscle and regulating muscle contraction.

Traditionally, worm and fly fishermen are often scornful of one another as the latter consider their fishing style to be more artistic and the former that theirs can catch more. However, versatile fishermen have a tackle-box full of bait for all conditions and know that flies also catch big fish. The S100A1₂/twitchin connection landed by Kemp *et al.* is a big catch for those who angle with worms; fishing with flies may land the rest of the story. □

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VISION

Resolving perceptual ambiguity

Jeremy M. Wolfe

IN 1761, M. DuTour placed a prism in front of one eye so that "different objects will be projected on corresponding portions of the two retinas". He goes on to describe what he saw, saying: "If such images could both be simultaneously perceived, a confused picture would be seen... but this was not what was evident to me in the test that I made: sometimes I would see only objects projected in the bare eye, sometimes only those in the eye covered by the prism, and sometimes the object projected in one would seem to intermingle with the objects projected in the other" (ref. 1).

On page 621 of this issue, Logothetis *et al.*², continuing their earlier work^{3,4}, report on a clever experiment that links this classic phenomenon of binocular rivalry to the broader issue of perceptual stability — how we perceive a single interpretation of an ambiguous perceptual world. Like DuTour, they show each eye a different stimulus. Their version of this binocular rivalry experiment shows just how stubbornly the visual system tries to infer a single, coherent interpretation from this biologically implausible input.

Before describing this new result, it is worth providing a bit more detail about the basic phenomenology of binocular rivalry. Consider stimuli like those used by Logothetis *et al.* The left eye sees lines

tilted to the left of vertical and the right eye sees lines tilted to the right. Let us suppose that these are relatively large stimuli (Logothetis *et al.* use stimuli that are 3 degrees of visual angle on a side —

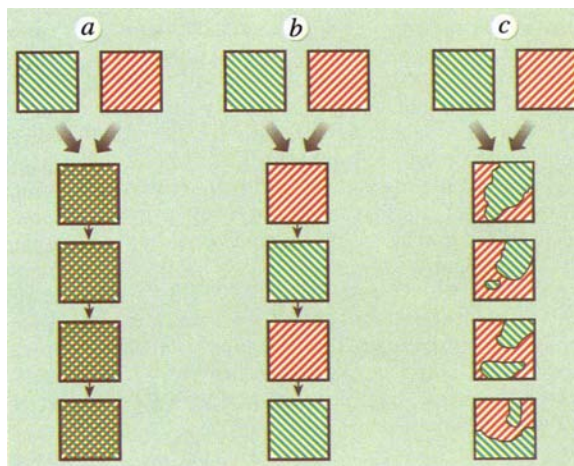


FIG. 1 What happens when the two eyes see two different stimuli? Under most circumstances, the two stimuli do not fuse (a), nor do the two monocular images rival as wholes (b). Rather, we see a continuous struggle between regions dominated by one eye's input and regions dominated by the other (c).

large enough for our purposes). Several possible outcomes are *not* seen under normal circumstances. As DuTour noted, two distinct monocular patterns do not fuse together (unless you flash the stimuli on and off at the correct rates⁵) (Fig. 1a). Nor is binocular rivalry a competition between the two eyes as a whole. This

would produce exclusive visibility of one or the other monocular stimulus — something that is not normally seen with stimuli larger than 1 square degree (ref. 6) (Fig. 1b).

In most examples of rivalry with large stimuli, what is seen over time looks something like the sequence in Fig. 1c. Each region of the retinal image in one eye seems to fight with the corresponding region in the other eye. However, these interocular battles are not completely local, oblivious to the tide of war elsewhere. Regions expand, contract and vanish in a seemingly systematic fashion. This evolving patchwork probably reflects the actions of two mechanisms: first, an interocular competition for dominance by the left eye or the right eye at each location, and second, a stimulus-specific mechanism that seeks to impose a single perceptual interpretation on the competing stimuli.

In the example shown in Fig. 1, the interocular mechanism would be choosing between the left-eye and right-eye stimuli, whereas the stimulus-specific mechanism would be choosing between left and right oblique gratings. These two choices are perfectly confounded in the usual binocular rivalry situation. Logothetis *et al.* have found a way to unconfound them. This allows us to see the stimulus-specific mechanism building a coherent percept without its being thwarted by the more local, interocular struggles for dominance⁶.

Logothetis and colleagues have taken a number of actions that probably serve to reduce the influence of the interocular

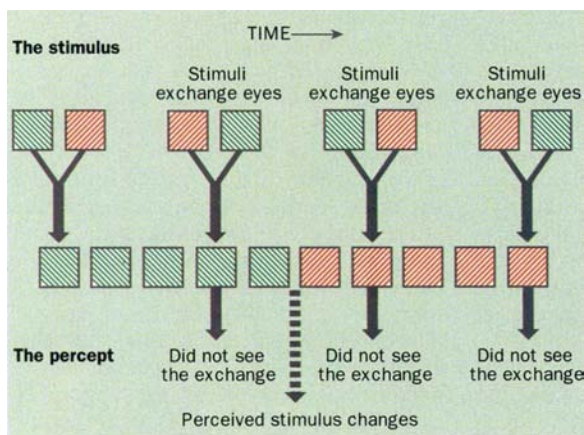


FIG. 2 Logothetis *et al.* present oblique bars to one eye and orthogonally oriented bars to the other. Three times a second, the stimuli exchange eyes. Under the conditions described in the text, observers do not see this exchange. Instead, the visual system tries to hold on to a stable percept, even if the input supporting that perception moves from the left to the right eye.

competition. What they did and saw is illustrated in Fig. 2. They used stimuli similar to those in Fig. 1. They flashed them on and off at 18 Hz to produce a continuous flicker. Their critical manipulation was to exchange the stimuli of the left and right eyes three times a second. Thus, looking with just one eye, you would see a flickering grating repeatedly changing orientation by 90°. Their surprising finding is that this exchange is not seen when both eyes are open.

This is a clear 'win' for the stimulus-specific mechanism. It is managing to preserve the perception of lines of one orientation, even when those lines shift from one eye to the other. If the interocular mechanism were more potent, you would see changes in the currently winning, dominant eye and so you would see the orientations change.

It is not entirely clear why the conditions used by Logothetis *et al.* favour the putative stimulus-specific mechanism. There are ways to eliminate rivalry entirely and produce the plaid pattern of Fig. 1a. Changing from high-contrast bars of black and white to low-contrast bars of very slightly different shades of grey will do it⁵. So will flickering the pattern on and off every fifth second or so⁶. The authors' stimuli are flickering gratings of 20 per cent contrast — not low or slow enough to eliminate rivalry, but perhaps an intermediate state that preferentially disrupts the interocular processes.

There is some evidence to support the idea that local interocular competition has been disrupted. Logothetis and colleagues report whole eye rivalry (Fig. 1b) with their 3 × 3 degree stimuli. Whole eye rivalry usually requires stimuli smaller than about 1 deg, so whole eye rivalry with such a large stimulus suggests reduced local interocular competition. In informal observations in my laboratory,

this dominance of stimulus factors over interocular factors is reduced if either the contrast is raised or the flicker is eliminated. Further parametric work is needed on this point.

If this were merely an illustration of some arcane details of binocular rivalry, it would be of only limited interest. However, its real value lies in its potential to bring under scientific scrutiny the stimulus-specific mechanism described above. Normal visual perception always represents the effort to impose a single interpretation on ambiguous visual input. As a slightly strained example, consider the full stop at the end of this sentence. It

could be a speck of dirt. It could be a small portal into a black void beyond the page. You see it as a full stop because your visual system renders the verdict that a full stop is the most plausible interpretation of that black spot in the image.

Every psychology text contains illustrations of what are called 'ambiguous' or 'reversible' figures — cleverly contrived cases that prevent the visual system from settling on a single interpretation. Examples include the Necker cube and Rubin's face-vase figure. In these cases, one stimulus produces two plausible perceptions. Binocular rivalry allows us to present two arbitrary competing stimuli to the visual system. The demonstration by Logothetis *et al.* enables us to minimize the usual interocular competition. This opens the way for experiments that pit one percept against another in order to uncover the rules that are used to resolve perceptual ambiguity. □

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Inner ferment

THE greenhouse effect is not a purely human invention. The world's cows and termites eat plants, and ferment their saccharides internally to carbon dioxide and the potent greenhouse gas methane. They emit about 300 million tonnes of it into the air each year. And, of course, the plants that evade the cows and termites still die sometime, and similar organisms degrade their saccharides, again to carbon dioxide and methane.

These fermentations proceed through a 'consortium' of many different microbes. The primary gaseous product is hydrogen. This is then seized by the methanogens and combined with carbon dioxide to give methane. For dairy farmers, methane is a pure waste product. They try to reduce it by feeding their cows with 'ionophores' — chelating agents that inhibit the methanogens by seizing the sodium and potassium ions they need for their hydrogen-uptake chemistry.

Curiously enough, we ourselves seem to have solved this problem. We all make hydrogen in our large intestine; even *Escherichia coli* can make hydrogen from saccharides, though some clostridia are better at it. But only a third of us take it on to methane. The rest of us somehow suppress our internal methanogens, and emit hydrogen from our rear ends.

Now some *Streptomyces* moulds put out natural ionophore chelating agents, which kill rival bacteria by stealing their potassium. The methane-free majority among us, says Daedalus, must harbour a bug that makes a similar inhibitor. So DREADCO biochemists are studying the internal flora of volunteers, to identify this organism and the inhibitor it uses. They plan to insert its crucial genes into promising members of the internal flora of cows and termites, and the free-living cellulose-rotting organisms. The new, improved organisms will displace and suppress the methanogens. Some of them may even put the hydrogen to better use — perhaps to make acetate.

When all is ready, DREADCO will release the new genetically engineered organisms into the environment. They will spread rapidly. Every cow and termite in the world will have a mighty internal spasm as the new internal regime takes hold. Thereafter, they won't produce methane, but hydrogen instead.

Environmentalists will agonize over the use of wicked genetic engineering to counter the wicked greenhouse effect. The rest of us will enjoy the benefits. Not only is hydrogen a non-greenhouse gas; it is widely acclaimed as the virtuous, non-polluting fuel of the future. Generated in fermenters and used to power fuel cells, it will be the ultimate renewable source of energy.

David Jones

The 'species alias' problem

SIR — May and Nee¹ correctly raise the issue of synonymy — multiple names for single biological entities — as an important one for biodiversity studies. As Solow *et al.*² showed, roughly 20% of names for thrips proposed during this century are now regarded as synonyms. Using the time needed to detect the synonymy to model asymptotic levels, true

relation between synonymy rate and mass (using order-of-magnitude values for each order) is $r_s=0.755$ ($P<0.05$). Rate estimates range from a low of 17% for the inconspicuous and poorly known shrew opossums (Paucituberculata) to 78% for tapirs (Perissodactyla; other groups, edentates 64%, opossums 64%, primates 59%, carnivorans 56%, ungulates 56%, monitos

systematic concepts used. Although future investigations will assuredly reveal many current names to be synonyms of others now in circulation, changing systematic concepts and improved technologies for detecting and defining species will continue to breathe new life into yesterday's entombed synonyms.

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NOMENCLATURE CHANGES FOR SPECIES OF NEOTROPICAL MAMMALS SINCE 1980⁷

Mammalian orders	(a)* Species both in 1982 and currently	(b) Synonyms newly sunk	(c) Synonyms newly elevated	(d) Names newly proposed
Didelphimorphia	52	21	9	3
Paucituberculata	5	2	0	1
Microbiotheria	1	0	0	0
Xenarthra	29	0	0	1
Lipotyphla	13	0	1	2
Chiroptera	236	6	23	11
Primates	46	1	29	7
Carnivora	54	0	4	0
Perissodactyla	3	0	0	0
Artiodactyla	17	0	2	0
Rodentia	479	32	105	35
Lagomorpha	2	0	0	0
Faunal totals	937	62	173	60

*Current species diversity estimate is the sum of columns (a) + (c) + (d)

synonymy rates may actually be closer to 40%. In discussing the general implications of this argument for biodiversity, May and Nee stated that rates of synonymy are lower among the more charismatic vertebrate groups, and that global estimates of diversity need to be correspondingly reduced to correct for these lacunae of misunderstanding.

Both of these important conclusions are debateable. Using an ongoing database for Neotropical mammals³, it is possible to consider these points for roughly 25% of the world's mammal species. Across 12 terrestrial mammal orders, 1,173 species and 1,439 subspecies are currently recognized as valid, with another 2,143 names treated as synonyms. So defined, synonyms comprise 45% of all mammalian names, about twice the fraction demonstrated for thrips (this number actually climbs to 75% if subspecies are discounted). Synonymy rates for mammalian orders are not correlated with ordinal diversity ($r_s=0.06$), but are positively (not negatively) correlated with body size and presumed conspicuousness. The rank cor-

relation between synonymy rate and mass (using order-of-magnitude values for each order) is $r_s=0.755$ ($P<0.05$). Rate estimates range from a low of 17% for the inconspicuous and poorly known shrew opossums (Paucituberculata) to 78% for tapirs (Perissodactyla; other groups, edentates 64%, opossums 64%, primates 59%, carnivorans 56%, ungulates 56%, monitos

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In the red zone

SIR — The problem of whether temporal fluctuations in natural populations are composed mainly of low- or high-frequency variation is of theoretical as well as practical interest for ecological and evolutionary questions. Cohen¹ examined eight population models given by difference equations of the form $P_{t+1} = P_t \cdot f(P_t)$, where P_t is the population density at time t , and $f(P_t)$ is a density-dependent fitness function. He argued that high frequencies dominate in the power spectra of chaotic time series obtained from these models. This casts doubt on the usefulness of one-dimensional difference equations for modelling fluctuating population dynamics, because low frequencies are expected to dominate the spectra of natural time series^{2,3}.

It may, however, be too early for general conclusions. Cohen¹ reports results for a limited choice of parameters in each model, yet these models exhibit chaos for a broad

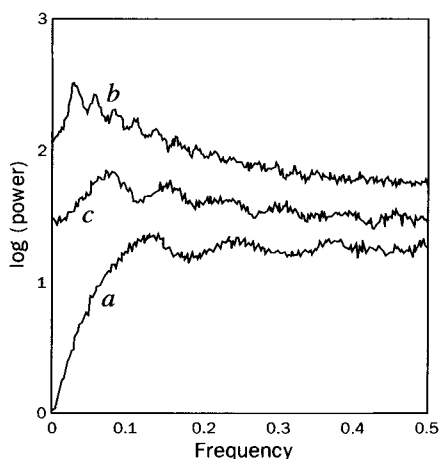


FIG. 1 Averaged power spectra for three one-dimensional models obtained using exactly the same method as Cohen¹. Curve *a* results from the Ricker equation, with $r=4.7$. With increasing r , the initial slope and the similarity with white noise increase, although the fluctuations in the corresponding time series become very large. Curve *b* results from the Maynard Smith model, with $a=0.02$, $r=1.5$ and $b=50.0$, and curve *c* from Bellows' equation with $a=0.1$, $r=3.0$ and $b=4.8$. Both spectra are reddened.

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range of parameters. Even in the simple Ricker model, with only one parameter in the fitness function (model 1 in ref. 1), the spectrum changes for different parameter values: increasing the growth rate r flattens the spectrum (Fig. 1, curve a), and it becomes reminiscent of white noise, where all frequencies have equal power.

More sophisticated models, with more parameters, are more flexible, and the changes can be more dramatic. For example, in the Maynard Smith model $f(P_i) = r \cdot [1 + (aP_i)^b]^{-1}$ (model 5 in ref. 1), the dynamics are determined by the parameter b , describing the type and strength of competition, and by the intrinsic growth rate r , whereas a scales the carrying capacity. In this model, large values of b (severe density dependence) and small values of r typically yield reddened spectra with more power at low frequencies (Fig. 1, curve b).

A very general model for density dependence, not analysed by Cohen¹, is Bellows⁴ fitness function $f(P_i) = r \cdot \exp[-(aP_i)^b]$. (The Maynard Smith model is a simplified version obtained by truncating a Taylor series expansion of the denominator.) In this model, reddened power spectra (Fig. 1, curve c) are common. An index for the colour of a spectrum is the ratio between the area under the spectrum ranging from 0 to 0.25 and

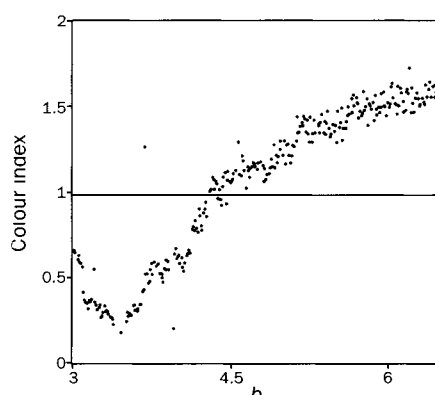


FIG. 2 Colour index for spectra from the Bellows model (see text). White noise has an index of 1 (horizontal line). Indices larger than 1 indicate reddened spectra and those smaller than 1 blue spectra. Outliers in the figure correspond to spectra with discrete peaks in the sense of Cohen¹.

the area ranging from 0.25 to 0.5: the ratios of blue spectra with more power at high frequencies are smaller than 1, while those of red spectra are larger than 1.

Figure 2 shows the colour index for the spectra corresponding to 300 values of parameter b in the interval [3, 6.5]. The other parameters were fixed at $r = 2.5$ and $a = 0.005$. Only b values leading to chaotic time series were considered. The lower

limit of the b interval was chosen to ensure that chaotic time series are possible, the upper limit to prevent unrealistically large fluctuations in these trajectories. Red spectra occur for a broad range of b values describing intermediate to strong competition.

We conclude from our calculations that blue spectra are indeed easy to observe in the models considered by Cohen. For each model, however, there is a range of parameters where fluctuations are chaotic but the corresponding spectra are reddened, or at least very similar to white noise. For the equations of Bellows and Maynard Smith, which are the most general one-dimensional ecological models^{4,5}, reddened spectra are common, especially when competition is intense. The open question posed by these results and by Cohen's is to understand when and why some parameter values give blue spectra and others red spectra.

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Why squid can breathe easy

SIR — Cephalopods are all predators and typically have high metabolic rates. Nevertheless, a huge biomass of squid apparently live in the deep oceans, often in severely hypoxic waters¹. This prompted us to examine the gills of various deep-sea cephalopods. Compared with their shallow-water relatives, squid from the cold deep oceans tend to have large gill areas and very thin blood–water barrier thicknesses² (see table). Among fish, only the warm-blooded tuna^{3,4} have similar weight-specific gill areas and comparable barrier thicknesses. The limited data available on the morphometrics of deep-water fish gills suggest that these gill areas are actually reduced in comparison

with shallow-water species⁵.

Estimates of the gill diffusion capacities of deep-water squid indicate that these animals could remain active, swimming about rapidly even in the oxygen-minimal layers of the open ocean. The price is very fragile gills. Cephalopods can tolerate these conditions where fish cannot because the gills do not form part of the buccal apparatus; there is no danger of damage from sharp fragments of cuticle, scales and bones penetrating the gills when the animal feeds. Deep-water squid are living in an environment where only very clear, grit-free water enters the mantle — even the anus is downstream of the gills. We suspect that very high oxygen

diffusion capacities of these fragile gills give squid like *Bathyteuthis* or *Brachio-teuthis* a much-needed edge in their competition with fish and are perhaps in part responsible for the huge numbers of cephalopods to be found in the deep oceans.

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CEPHALOPOD GILL DATA

Cephalopod species	Weight (g)	Ambient seawater oxygen content (ml per l)	Gill area (cm ² per g)	Blood–water diffusion barrier (μm)	Gill diffusion capacity (ml O ₂ per kg per mmHg per min)
Deep-water species					
<i>Bathyteuthis abyssicola</i> ²	4.27	2.59	21.88	1.10	2.999
<i>Brachio-teuthis riisei</i> ²	8.26	3.23	45.14	1.01	3.160
<i>Cranchia scabra</i> ²	11.51	3.73	27.04	3.10	0.755
<i>Liocranchia reinhardtii</i> ²	5.49	2.59	13.33	1.58	0.882
<i>Histioteuthis reversa</i> ²	37.09	4.44	11.87	2.52	0.435
<i>Octopoteuthis danae</i> ²	26.53	3.43	8.96	1.63	0.492
<i>Vampyroteuthis infernalis</i> ²	18.81	3.62	4.74	3.34	0.311
Shallow-water species					
<i>Octopus vulgaris</i> ⁶	40.00	5.15	5.40	8.58	0.058
<i>Eledone moschata</i> ²	334.00	5.15	2.91	14.98	0.025
<i>Alloteuthis subulata</i> ⁶	8.98	5.15	11.76	6.19	0.274
<i>Lolliguncula brevis</i> ²	10.58	5.15	7.03	1.34	0.512

Utopian eugenics

John Harris

The Lives to Come: The Genetic Revolution and Human Possibilities. By Philip Kitcher. Simon and Schuster/Allen Lane: 1996. Pp. 381. \$25, £20.

At least seven books have been recently published examining ethical dilemmas provoked by the genetic revolution. The latest of these, by Philip Kitcher, is stimulating, informative and courageous. Like Jonathan Glover's trail-blazing study *What Sort of People Should There Be?* (Penguin, 1984), Kitcher's book concentrates on the use of the genetic revolution to influence the future both for individuals and for humanity. One of the book's great strengths is that it does not simply identify and re-pose the ethical and social questions raised by our ability to use and abuse genetic technology, it also attempts to elaborate the principles and approaches that help us to answer these questions.

Of all the books that have so far appeared on this topic, Kitcher's is certainly the best informed scientifically and by far the best illustrated with examples, cases and predicaments drawn from real life and real science. In so far as I am able to judge as a nonscientist, the scientific background is excellently and clearly presented, and the detail is sufficient to convey real insights into the scope and limits of the new technology and to impart a real understanding of the scientific basis of the dilemmas it poses. The early chapters of the book describing the basic science, the possible therapies and manipulations and the agonizing dilemmas — both personal and social — of testing are written in a humane, lucid and informative style that is a pleasure to read and to learn from.

If I have one small caveat about the first half of the book, it is the almost throwaway treatment of the dilemmas of HIV testing. Having pointed out that the psychological pressures on women make it hard, perhaps impossible, for them to reach decisions that are in the best interests of the fetuses they carry, Kitcher comments that "poor women who resist testing for AIDS would surely prefer, other things being equal, to save their babies from suffering an early death.... Compelling them to be tested and to take AZT may be the right choice as a stopgap measure, since it may be a choice that saves the child, but it is only part of a complete response to the problem." Why Kitcher thinks this is the right choice even

as a stopgap measure is unclear. It is now generally accepted that the risks of vertical transmission of AIDS are low, somewhere between 15 and 30 per cent, perhaps less if measures are taken to reduce the risk at delivery and if breastfeeding is avoided. Because the long-term effects of AZT on children are unknown, it is far from clear that it is in the interests of the child to subject it to AZT during

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Does the loss of our genetic innocence commit us to some form of eugenics?

pregnancy when there is a reasonable chance that it will in any event be born free of the disease.

The book is nicely divided by an interlude, and in the second half Kitcher turns to the issue of eugenics and begins to consolidate his defence of what he terms "utopian eugenics", which broadly speaking champions the moral legitimacy of the enterprise of choosing our descendants. He points out that once "we lose our genetic innocence, we are committed to some form of eugenics". This is because when we have the power to choose, we exercise that power as much by our decisions not to intervene and make changes as we do by more direct interventions.

As soon as it is clear that the relevant ethical question is not whether but how to practise eugenics, the problem of our responsibility to and for future generations takes on a quite different character. A crucial issue is whether there is a way of dividing legitimate from illegitimate eugenic practices. Kitcher suggests that we must look to the issue of the quality of the lives we create and that responsible people faced with the bare choice of continuing or terminating a pregnancy will, where the quality of the life-to-be is likely

to be poor, decide to terminate the pregnancy. Kitcher analyses quality of life along three dimensions and all of them raise difficulties for his account. But before I explore these, I should point out one important drawback of his book.

Kitcher concentrates throughout on abortion as the most important and most likely method of controlling which individuals will or will not come into existence. Although this may be true in most cases and in the short-to-medium term, it rather distorts the argument. We find him saying things such as "using amniocentesis to select for sex or curly hair seems morally dubious to say the least"; but this is because, after amniocentesis, the only method of using its results to influence the sorts of people that will be born is abortion. It seems far less obvious that it is morally dubious to use pre-implantation screening of embryos, or manipulation of gametes, to select for what I would call 'morally neutral' traits such as gender or curly hair, particularly where not all *in vitro* embryos can be implanted in any event. In this way, Kitcher has conflated the issue of the ethics of determining the various traits that future people will possess *per se* with the question of the ethics of doing this by abortion. Because abortion is, for a whole range of reasons, a far more morally consequential act than selection of gametes, we are prevented from separating the question of the ethics of choice of traits from the question of the ethics of abortion.

Considering the quality of a human life, Kitcher distinguishes three different dimensions to its evaluation. The first involves the capacity to develop a sense of what is important in life, the second assesses the extent to which desires central to the person's life plan are satisfied and the third is concerned with the character of the person's experience, the balance of pleasure over pain. An acute problem involved in using quality of life as a measure of the value of life, as Kitcher does, is that once these ideas are conflated then it follows that lives with more quality are more valuable. This not only creates an imperative for utopian eugenics, which Kitcher accepts, but also leads to the conclusion that the premature death of someone with a less valuable life is less tragic and less undesirable than that of someone with a more valuable life. From this it follows that the wrongness of murder, for example, varies with the quality of life of the victim. It also seems to imply that in conditions of scarce resources we should prioritize the allocation of resources to those whose lives are

Peter Menzel/SPL

of greater quality.

But Kitcher should not be blamed if he has found difficulties in developing a positive account of the value of life and how it relates to decisions about which lives to create and sustain. These are problems that no-one has fully resolved. Kitcher's arguments are always compassionate, always ingenious and always worth pursuing. The book is refreshingly direct and honest, well illustrated with real examples presented in sufficient detail for a full understanding of the dilemmas they raise, and is a more than welcome addition to what will undoubtedly be a growing literature on these important topics. □

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Uncertain futures

Crispin Tickell

One World: The Health and Survival of the Human Species in the 21st Century. Edited by Robert Lanza. *Health Press, Santa Fe, New Mexico: 1996. Pp. 325. \$25.*

ROBERT Lanza has put together an ambitious compendium of short essays about the human condition in the next century. With millennial thoughts in many people's minds, it is a good time to do so. Other societies and civilizations, indeed other animal species, have greatly affected the natural environment around them. But we are perhaps the first human generation to be able to see and assess the global consequences of our actions on the land, waters and atmosphere of our planet.

Since the Industrial Revolution got under way some 250 years ago, and humans began to multiply at their present giddy-making rate (over 90 million more people every year), change has accelerated, sometimes steadily, at other times in bewildering spurts. In the past, generations could communicate with each other over the years on the basis of a slowly evolving but common vocabulary, common experience and common outlook. That is no longer so. With sharp discontinuities behind and before us, uncertainties have greatly increased and prediction has become more hazardous than ever.

It cannot be said that most of the contributors to this book say much that is new or interesting. They constitute an impressive array of the great and the good and sometimes powerful, from the secretary general of the United Nations and the heads of certain of its agencies to prominent scientists, doctors and a selection of ministers of health. A much used

word throughout the book is 'challenge'. It seems usually to mean inability to cope, or in some cases to understand what is happening. It begs the question of whether the authors are so great and good after all.

The contributors fall into four main categories. There are those from the ruling establishment who state the worthy, the obvious and the well meaning. The hand of the speech-writer and public-relations man is visible from time to time. If some of these eminent persons had really thought through and written their essays themselves, the result would not have been like this (or let us hope not). There are those preoccupied with the short term, in particular those involved in medicine, who struggle with the worldwide problem of rising health costs and changing patterns of disease. Many in this group tend to see the future as an extrapolation of the present. Next there are a few with endearing bees in their bonnets who at least stand out for being different and in one case for some colourful waffle. Here also are one or two with outsized egos. Finally there are those with some-

thing genuinely original to say. Among them are Linus Pauling, Christian Barnard and Carl Sagan as dissenters from the prevailing wisdom, Robert Gallo, Jonathan Mann and Luc Montagnier on AIDS; and W. Harding Le Riche for an analysis that constitutes a cry of pain at what we are doing to the world, and what may come of it.

Essays of this kind are far harder to write than a full-length book. The task set was almost too difficult. Even so, few manage to get their minds around the issues as a whole, or see each issue in terms of the others. Prediction may be impossible, but a greater sense of interconnectedness, of wider ranging across the disciplines, would have shed more light on the prospects ahead. One thing is sure. The future will not be as anyone expects: less upwards and onwards, more sideways in unfamiliar directions, and even sometimes downwards or backwards. Ten years into the next century, this book will be a period piece. □

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CRACKING up — The San Andreas fault taken from *The Viking Historical Atlas of the Earth* edited by Roger Osborne and Don Tarling. Using full-colour maps to reconstruct the changing geography of the Earth, the atlas combines scientific data with a non-technical approach. Viking, £20.00.

Frames of thought

Basileios Drolias

Mach's Principle: From Newton's Bucket to Quantum Gravity. Edited by Julian Barbour and Herbert Pfister. Birkhauser: 1995. Pp. 536. \$64.50, DM 118, Sfr98.

ERNST Mach is famous for his controversial statement of the principle of inertia. He introduced it at the end of the nineteenth century as a way of rationalizing Newtonian physics. Despite rationalizing the Universe with three laws, Newton left behind the concept of 'absolute space', which seemed far removed from human experience. Mach stated his principle in *The Science of Mechanics: A Critical and Historical Account of Its Development*, in which he dealt with the definition of inertial frames. For Newton, an inertial frame was one at rest or moving with constant velocity with respect to absolute space. But according to Mach, inertial systems are defined with respect to all the masses in the Universe. In another formulation, Mach's principle states that inertia is an effect of all the masses in the Universe.

Mach believed that physics should be kept separate from mysticism and should always be concerned with meaningful matters related to experience; his explanation of inertia and his dismissal of absolute space in favour of relativism are expressions of that belief. But it is not clear whether Mach intended his thesis to be a starting point for a new theory of mechanics or just a clarification of the problem of inertial frames. What is certain is that Mach's idea was not very popular among the natural philosophers of his day, at least, that is, until Einstein championed the principle by claiming that it was one of the main influences behind his general theory of relativity. (It was Einstein who named it Mach's principle.) Einstein was more than a follower of Mach; he imposed his own ideas on the principle and persuaded contemporary physicists to become interested mainly in the 'Einstein-Mach' principle or, to put it differently, only Einstein's version of Mach's principle.

Unfortunately, however, Einstein misunderstood the principle as embodied in general relativity. And despite his great admiration for Mach and his idea, Einstein received nothing but harsh criticism of general relativity from Mach. Einstein struggled for years to incorporate Mach's principle into general relativity, convinced that his theory was totally Machian, or should at least be made so. It was this conviction that forced him to introduce the cosmological constant into his equations, albeit with-

out success.

Einstein's strong belief in Mach's principle brought it to the peak of its popularity early this century, even though Einstein would in later life renounce it. Because of the principle's vagueness and Einstein's various formulations of it, it exists today in a multitude of versions, each somehow related more or less to Mach's original idea of defining inertial systems. Not surprisingly, it is remarkably difficult to obtain a clear view of what modern researchers think about the principle and how they apply it to their work.

This book is based on a conference on Mach's principle held in Tübingen in

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Ernst Mach believed that physics should be concerned with meaningful matters related to experience.

1993. In addition to the papers presented by the participants and edited transcripts of the discussions that followed, it includes extracts from relevant papers by scientists of the early twentieth century such as Mach, Einstein, Schrödinger, Poincaré and others. The noticeable lack of opposition to Machian ideas might mislead the unwary reader into thinking that they have won an undisputed berth among scientists. Nevertheless, the book succeeds in giving an excellent view of these ideas from perspectives as diverse as those of the historian, the philosopher, the experimentalist and the theorist, and clearly shows how they relate to current research in cosmology, quantum gravity and general relativity.

As with most symposium volumes, the quality and level of the papers is variable, but in general most of them are proficient and fairly advanced, requiring at least a knowledge of general relativity (only the first few historical chapters will

be readily accessible to a nontechnical reader). The edited discussions, although fairly dense at times, graphically convey the spirit of the conference. And to avoid misunderstanding and confusion, the authors helpfully say in their papers which particular version of Mach's principle they are talking about.

There has until now been a paucity of detailed treatises on the Machian approach in mechanics. Further, the Machian idea is still not part of the standard framework of interpretation of general relativity, although work on the topic by J. A. Wheeler and J. Isenberg is well worth exploring. The book will not completely plug the gap in the literature but it should provide a stepping stone for a debate that will help gain a better understanding of what Mach described as "a Universe which is given to us only once". □

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All-encompassing

James L. Gould

Magnetic Orientation in Animals. By R. Wiltschko and W. Wiltschko. Springer: 1995. Pp. 297. DM190, £92, \$149.

THE Wiltschkos are respected pioneers in the study of animal orientation. They provided the first really convincing evidence of magnetic field orientation in migrating birds, and, through a series of clever and well-controlled experiments, have painstakingly pieced together a nearly seamless picture of the elaborate development of orientation ability and strategies in homing pigeons.

They have set out in their newest book — one of the slim, densely packed volumes in Springer's 'zoophysiology' series — to review magnetic compass orientation in animals. The result is a wide-ranging and sometimes over-enthusiastic review of compass orientation. Their blasé treatment of the early and well-justified scepticism about much of this work — the need to average data through three steps in just one of three possible sequences, or to use cages with radial rather than tangential perching monitors, and so on — will worry readers who still harbour doubts about the complete reality of magnetic field orientation.

The general presentation is clear but very concise, and seriously under-illustrated. More is presupposed than many readers may bring to the book — a knowledge of the pattern of polarized light in the sky, for instance, or the Sun's course as a function of latitude and season, or the



HOLE in the wall gang — A colony of carmine bee-eaters from the tropics of Africa. Colonies can be as large as 10,000 pairs and migrate towards the equator in winter. Taken from the *Collins Atlas of Bird Migration* edited by Jonathon Elphick. HarperCollins, £16.95.

singlet versus triplet states of electrons.

And although the Wiltschkos are refreshingly critical in their treatment of some work, in other cases they are ambivalent at best, seeming to accept at face value work that, when read carefully, inspires (at least in me) very little confidence. Take the notorious case of 'human homing'. We are told about the claims for this ability, but warned that virtually all attempts at replication have failed. Oddly enough, none of the dozens of failures is cited in the otherwise extensive bibliography; instead we are referred to a 'review' by the main proponent of this human homing. The authors conclude that the alleged ability is vague at best, but later tell us that a magnetic compass has been clearly demonstrated in humans.

The book is at its best in dealing with the development and interaction of the many cues involved in orientation. It is excellent when cases are treated in detail, as with eels, salmon and whales. It is a pity that pigeons and migrating birds could not be discussed in the same depth — although the breadth of the treatment of birds is nevertheless truly astonishing.

The other high point of the book is the balanced and generally well-informed discussion of possible detection mechanisms. Now that magnetic orientation and the development of orientation

behaviour have been worked out in so many species, the question of how the magnetic information is converted from one form to another and processed becomes increasingly important. The Wiltschkos make clear that there are at least three different strategies that have evolved, with more than one at work in at least some species. But the data so far are rather indirect, and the authors' almost palpable impatience with physiologists is easy to understand.

The other notable lapse in our understanding is the basis of the map sense of many species. The authors valiantly review the vast and utterly confusing literature on this subject. They show that for every experiment there is an equal and opposite experiment. Nature, I have always supposed, is simple at heart — how else could we hope to understand its ways? But the map sense seems to have been designed with malice and spite, in hopes of drawing the unsuspecting researcher into a foggy quagmire from which there is no apparent escape. Yet surely there is simplicity at the heart of this endlessly contradictory mass of data; the one certainty is that the Wiltschkos' book is the definitive place to begin the quest. □

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New in paperback

Evolutionary Concepts in the Nineteenth Century: Natural Selection and Patrick Matthew by W. J. Dempster. Pentland, £12.50. The story of Patrick Matthew and his ideas, which included the detailing of the principles of natural selection almost 30 years before Darwin.

Ecology: Individuals, Populations and Communities by M. Begon, J. L. Harper and C. R. Townsend (3rd edn). Blackwell Science, £24.95. "All ecology teachers and students would do well to sample this *nouvelle cuisine*", wrote R. H. Smith of the first edition in a review in *Nature* 319, 809 (1986).

Population Ecology: A Unified Study of Animals and Plants by M. Begon, M. Mortimer and D. J. Thompson (3rd edn). Blackwell Science, £18.50. An updated edition of the successful and concise undergraduate text.

Concise Lexicon of Environmental Terms edited by M. Grant and R. Hawkins. Wiley, £19.99. An Environmental Law Series book, published in association with the UK Environmental Law Association.

Biology of Freshwater Pollution by C. F. Mason. Longman, £22.99. A text for students of aquatic biology, limnology and environmental science and for scientists in the water industry.

Molecular Systematics edited by D. M. Hills, C. Moritz and B. K. Mable (2nd edn). Sinauer, £35.95.

Controlling Our Reproductive Destiny: A Technological and Philosophical Perspective by Lawrence J. Kaplan and Rosemarie Tong. MIT Press, \$25.

Chaotic Dynamics: An Introduction by G. L. Baker and J. P. Gollub (2nd edn). Cambridge University Press, £37.50, \$70.00 (hbk); £13.95, \$24.95 (pbk). A quantitative introduction to chaos and nonlinear dynamics for undergraduates.

Physical Metallurgy by Peter Haasen (3rd edn). Cambridge University Press, £80, \$125 (hbk); £29.95, \$44.95 (pbk).

The Scientific Companion: Exploring the Physical World with Facts, Figures, and Formulas by Cesare Emiliani (2nd edn). Wiley, £13.99. An introduction to the physical sciences: physics, astronomy, chemistry, geology, meteorology, biology, atmospheric science and oceanography. Written for the layperson or student.

Crystal structure of a mammalian phosphoinositide-specific phospholipase C δ

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Mammalian phosphoinositide-specific phospholipase C enzymes (PI-PLC) act as signal transducers that generate two second messengers, inositol-1,4,5-trisphosphate and diacylglycerol. The 2.4-Å structure of phospholipase C δ 1 reveals a multidomain protein incorporating modules shared by many signalling proteins. The structure suggests a mechanism for membrane attachment and Ca²⁺-dependent hydrolysis of second-messenger precursors. The regulation and reversible membrane association of PI-PLC may serve as a model for understanding other multidomain enzymes involved in phospholipid signalling.

PHOSPHOINOSITIDE-SPECIFIC phospholipase C (PI-PLC, EC 3.1.4.11) isozymes play a central role in most signal transduction cascades, including those regulated by G-protein-coupled and tyrosine kinase-linked receptors (for reviews see refs 1–3). In response to various extracellular signals, these isozymes generate two second messengers, D-*myo*-inositol-1,4,5-trisphosphate (InsP₃) and *sn*-1,2-diacylglycerol (DAG), by catalysing the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdInsP₂). Whereas InsP₃ elevates the intracellular calcium level⁴, the membrane-resident diacylglycerol activates many protein kinase C isozymes⁵. This bifurcated signalling pathway exerts control on many cellular responses including cell growth, proliferation, contraction, excitation and secretion.

There are three main classes of mammalian PI-PLCs: PLC- β , PLC- γ and PLC- δ (ref. 2). The δ -isozymes are smaller (relative molecular mass 85,000; *M_r* 85K) than PLC- β and γ (*M_r* 150K). The widespread appearance of δ -type homologues in plants⁶, yeast⁷ and slime moulds⁸ suggests that this archetypal enzyme class evolved rather early in eukaryotes. PLC- δ shares similarity with the other two classes throughout its sequence, but most prominently in two regions, which have been denoted X and Y (ref. 2). In comparison to PLC- δ , the PLC- β isozymes contain an additional regulatory carboxy-terminal region that is responsible for the specific binding and activation by G-protein α_q subunits⁹. In the γ isozymes, an internal SH2/SH3 domain array inserted between the X and Y regions is involved in regulation by tyrosine kinase-linked receptors¹. All mammalian PI-PLCs require calcium for activity³; at physiological calcium concentrations, they prefer PtdInsP₂ and phosphatidylinositol-4-phosphate (PtdInsP) over phosphatidylinositol (PtdIns) as substrates¹⁰. In contrast, prokaryotic PI-PLCs (*M_r* 35–37K) are secreted, metal-independent, and hydrolyse only PtdIns and its analogues, but not PtdInsP or PtdInsP₂ (ref. 11).

We report here the three-dimensional structure of a catalytically active deletion variant of the rat δ 1-isozyme at 2.4-Å resolution. PLC- δ 1 consists of four domains, from N to C terminus: (1) a PH domain that is absent in the deletion variant used for the structure determination reported here, but which was recently described¹²; (2) a helical EF-hand domain; (3) a catalytic TIM-barrel (triosephosphate isomerase-like) domain; and (4) a C-terminal β -sandwich domain that has a structure similar to the first C2 domain of synaptotagmin I. This multidomain structure is likely to be common to all mammalian PI-PLC isozymes.

Structure determination

The crystals of PLC- δ 1 have cubic space group *F*₄32 with unit cell dimensions *a* = *b* = *c* = 398 Å, and diffract to 2.3-Å resolution. The crystals have two monomers per asymmetric unit with 70% solvent content. The structure was solved by multiple isomorphous replacement (MIR) and refined to an *R*-factor of 23.1% and a free *R*-factor of 29.1% for data between 8.0 and 2.5 Å.

To understand the nature of enzyme–substrate interactions, PLC- δ 1 crystals were soaked with InsP₃ and calcium chloride. The InsP₃/Ca²⁺/PLC- δ 1 complex was refined to 2.4-Å resolution (Table 1). By a modified soaking procedure, we obtained a 3-((3-cholamidopropyl) dimethylammonio)-2-hydroxy-1-propane-sulphonate (CHAPSO) detergent/enzyme complex (Table 1).

Molecular structure

The PLC- δ 1 structure has overall dimensions of 95 × 55 × 50 Å (Fig. 1b). The three domains are connected by two extended peptide stretches (residues 282–298 and 607–625) that form tight interactions with the domains. The areas in the interdomain interfaces vary widely. Whereas the EF-hand domain is not in contact with the catalytic domain, the C2 domain has extensive interfaces with both the catalytic and EF-hand domains.

EF-hand domain

The N-terminal domain (residues 133–281) consists of four consecutive EF-hand motifs with their characteristic helix–loop–helix topology¹³ (Figs 2, 3a; nomenclature for α -helices in EF-hands 1–4: E1 α /F1 α to E4 α /F4 α). Multiple EF-hands are found not only in small proteins such as calmodulin or troponin C, which consist exclusively of EF-hands, but also as parts of multidomain proteins. Resembling the EF-hand arrangement in calmodulin or troponin C, the PLC- δ 1 EF-hand units are pairwise distributed in two lobes (Fig. 3a). EF-hand 1 is only partly defined owing to missing electron density for E1 α and part of the loop between E1 α and F1 α . The interhelical angle of 128° between E2 α and F2 α is similar to the Ca²⁺-free conformations of EF-hands in calmodulin or troponin C^{14–16}. The interhelical angles of 115° and 94° for EF-hands 3 and 4 in the second lobe correspond more closely to the Ca²⁺-saturated state of calmodulin^{15,17}, despite the apparent absence of bound calcium. Indeed, the site of interaction between this lobe and the C-terminal domain includes a region where structurally equivalent residues in calmodulin in the Ca²⁺-

TABLE 1 Statistics for crystallographic data collection and phase determination

Diffraction data		X-ray source and wavelength (Å)		Concentration (mM)	$d_{\min}$ (Å)	Measurements	Unique reflections	Completeness (%)	R_{merge}^* (%)			
Datasets												
Native (I)	9.6	0.87			3.0	284,052	53,727	98.5 (99.0)	6.0 (19.6)			
Native (II)	BW7B	0.86			2.4	420,859	103,157	98.5 (95.3)	6.1 (27.9)			
PIP (I)	CuK $_{\alpha}$	1.54	1.0		4.5	57,168	15,499	98.7 (98.5)	10.8 (15.6)			
PIP (II)	9.6	0.87	1.0		3.7	106,035	28,559	96.4 (99.0)	5.7 (14.9)			
K $_3$ UO $_2$ F $_5$ (I)	CuK $_{\alpha}$	1.54	20.0		5.0	56,750	12,762	99.0 (99.7)	9.8 (16.1)			
K $_3$ UO $_2$ F $_5$ (II)	9.6	0.87	10.0		3.3	129,723	35,415	98.4 (98.5)	6.9 (22.4)			
NdCl $_3$	9.6	0.87	1.0		4.5	40,223	15,757	95.4 (95.0)	6.3 (10.9)			
K $_2$ PtBr $_6$	9.6	0.87	0.5		4.5	56,296	15,572	97.4 (95.8)	4.8 (7.9)			
UO $_2$ (Ac) $_2$	CuK $_{\alpha}$	1.54	20.0		5.0	67,453	12,125	98.0 (98.3)	8.0 (12.0)			
InsP $_3$ + Ca $^{2+}$	BL4	0.89	25.0 ± 1.0		2.3	755,288	113,383	96.4 (94.9)	6.2 (37.0)			
LaCl $_3$	BL4	0.89	1.0		2.6	388,360	81,102	98.5 (90.2)	4.2 (17.0)			
CHAPSO	BL4	0.89			2.4	402,870	84,771	88.7 (75.8)	6.4 (33.8)			
Phase determination												
Phasing power§												
Derivatives	R_{iso} (%)†	Sites	$R_{\text{Cullis}}^{\ddagger}$	12 Å	8.8 Å	6.9 Å	5.7 Å	4.8 Å	4.2 Å	3.7 Å	3.3 Å	Total
PIP (I)	27.9	7	0.75	1.18	1.29	1.65	1.86	1.49	1.30			1.51
PIP (II)	29.4	7	0.85	1.09	1.08	1.32	1.47	1.10	0.84	0.75		0.95
K $_3$ UO $_2$ F $_5$ (I)	25.7	7	0.72	1.58	1.71	1.98	2.12	1.65				1.84
K $_3$ UO $_2$ F $_5$ (II)	22.3	7	0.77	1.32	1.57	1.75	1.97	1.53	1.16	1.10	1.10	1.28
NdCl $_3$	34.9	4	0.92	0.91	0.79	0.89	0.89	0.67	0.55			0.73
K $_2$ PtBr $_6$	25.5	7	0.89	0.82	0.83	1.02	1.07	0.83	0.70			0.88
UO $_2$ (Ac) $_2$	34.1	6	0.84	1.27	1.12	1.23	1.38	0.98				1.17
Figure of merit				0.81	0.79	0.78	0.76	0.66	0.45	0.35	0.25	0.47
r.m.s. deviations¶												
	Resolution (Å)	Reflections ($F > 0\sigma$)	Total number of		R -factor/ R_{free} (%)		Bonds (Å)		Angles (°)			
			Atoms	Waters								
Native (II)	8.0–2.5	84,609	9,123	601	23.1/29.1		0.014		1.412			
InsP $_3$ + Ca $^{2+}$	8.0–2.4	93,045	9,136	585	23.3/28.6		0.013		1.354			

Crystallization. The $\Delta(1-132)$ deletion variant of rat PLC- $\delta 1$ was expressed and purified as described²⁰. The absence of an N-terminal PH domain in this variant does not affect catalytic activity in PLC- $\delta 1$ when assayed under conditions that measure non-processive catalysis. Crystals of PLC- $\delta 1$ were grown at 12°C in hanging drops by vapour diffusion equilibration against reservoirs of 1.4 M ammonium phosphate, pH 4.75, starting from 2.5 μ l protein at 10 mg ml $^{-1}$ in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5 mM EGTA, 1.0 mM DTT, 0.01% w/v sodium azide, 0.1% w/v CHAPSO, and 2.5 μ l reservoir solution. Typical crystal sizes were 0.4 × 0.3 × 0.3 mm 3 . Heavy-atom soaks were performed in freezing solution composed of 34% w/v PEG400 and 0.4 M sodium acetate, pH 5.65. CHAPSO was found to be essential for the growth of high-quality crystals. For the analysis of CHAPSO binding, the crystals were soaked for only 3 min before freezing; otherwise, crystals were presoaked for at least 2 h to wash detergent out of the crystals. **Diffraction data.** Because of the high radiation sensitivity of PLC- $\delta 1$ crystals, X-ray data were collected from single frozen crystals at 100K using the Oxford Cryosystem. All data were collected on 300 mm diameter MAR Research imaging plate systems. Images were recorded as 0.5° oscillation frames. For derivative data of K $_3$ UO $_2$ F $_5$ (I), di- μ -iodobis(ethylenediamine)-diplatinum (II) nitrate (PIP (I)) and UO $_2$ (Ac) $_2$, an Elliot GX11 generator (60 mA, 40 kV) in conjunction with a Supper double-mirror system was used as the CuK $_{\alpha}$ X-ray source. Other derivative data and the native dataset I were collected on station 9.6 at the Synchrotron Radiation Source (SRS), Daresbury, UK. Native dataset II was recorded at beamline BW7B of the EMBL outstation Hamburg, DESY; the complexes with InsP $_3$ and calcium chloride, lanthanum chloride and CHAPSO were measured at beamline BL4 of the EMBL outstation Grenoble, ESRF. The imaging plate data were reduced with the MOSFLM program package³⁹ and further processed with various CCP4 programs⁴⁰. **MIR phasing.** Two major heavy-atom sites for each of the PIP and K $_3$ UO $_2$ F $_5$ derivatives were determined by automatic Patterson interpretations as implemented in SHELXS-90 (ref. 41). The remaining heavy-atom sites in these and the other derivatives were located by difference Fourier techniques. The presence of anomalous signals in the synchrotron data delineated conclusively the correct handedness of the structure. Heavy-atom sites and MIRAS phases based on all derivative data sets were refined with MLPHARE⁴⁰. **Phase extension and density modification.** Solvent flattening for phase refinement and extension to 3.0 Å was performed with the program SOLOMON⁴². The solvent content was determined to be 70% after electron density was found to be consistent with two molecules of PLC- $\delta 1$ in the asymmetric unit. In total, 46 SOLOMON steps were applied starting with phases up to 3.8 Å and adding stepwise diffraction data to 3.0 Å. The R -factor decreased from 50% to 12% during this procedure. **Model building and refinement.** The polypeptide chains for both copies of PLC- $\delta 1$ found in the asymmetric unit were fitted to the solvent-flattened electron-density map (Fig. 1a) using the graphics program O⁴³. No continuous main-chain density could be observed for the N-terminal regions A133–A205 and B133–B160, and for the intervening regions between X and Y, A443–A486 and B446–B483. The N-terminal portion B161–B202 was initially modelled as polyalanine owing to uncertainties in the sequence assignment. The initial R -factor for this model was 41.2% for data between 15.0 and 3.0 Å. Further positional refinement in TNT-5E⁴⁴ with tight non-crystallographic symmetry (n.c.s.) restraints and full assignment of the N-terminal portion of molecule B resulted in a final R -factor of 23.1% (free R -factor, 29.1%) for data between 8.0 and 2.5 Å. The structural model is more complete for molecule B, for which residues B133–B157, B446–B483, B511–B513 and B649 are disordered. Molecule A shows more disorder at the N terminus (residues A133–A199, A443–A486, A511–A513 and A649), probably because there are fewer crystal packing restraints. Apart from the greater N-terminal disorder of molecule A, the two molecules are very similar. The refined model was used as an initial model for difference Fourier analyses of InsP $_3$ /Ca $^{2+}$ and CHAPSO complexes. The InsP $_3$ /Ca $^{2+}$ complex was refined to an R -factor of 23.3% (free R -factor, 28.6%) for data between 8.0 and 2.4 Å. We believe this to be the first protein ever solved in space group $F4_32$.

* $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$; numbers in parentheses correspond to the highest resolution shell.

† $R_{\text{iso}} = \sum_i |F_{\text{deriv}} - F_{\text{native}}| / \sum_i |F_{\text{native}}|$.

‡ $R_{\text{Cullis}} = \sum_i |F_{\text{PH}} \pm F_{\text{P}} - F_{\text{H,calc}}| / \sum_i |F_{\text{PH}} \pm F_{\text{P}}|$.

§ The phasing power is defined as the ratio of the r.m.s. value of the heavy-atom structure factor amplitudes and r.m.s. value of the lack-of-closure error.

|| R -factor = $\sum |F_o - F_c| / \sum F_o$; R_{free} calculated with 4% of the data.

¶ The r.m.s. deviations for bond angles and lengths relate to Engh and Huber parameters⁴⁵.

saturated conformation bind a helical peptide from a target protein¹⁸. However, the interface with the C-terminal domain is less extensive. From this first description of EF-hands within a multidomain protein, it is apparent that the interdomain interactions resemble the intermolecular interactions formed by calmodulin.

The loop regions of EF-hands 3 and 4 do not have typical calcium ligands, and indeed we have not observed metal binding in these regions; in contrast, EF-hands 1 and 2 contain calcium ligands. Preliminary analyses of complexes with calcium analogues suggest metal binding in at least the first EF-hand loop (data not shown). From our structure and from mutational analysis¹⁹, the second lobe is likely to serve a critical structural role. So far, there is no experimental evidence¹⁹ for a regulatory role of calcium-binding in the first lobe. Secondary structure predictions for other PI-PLC isozymes suggest that they too are likely to have a helix-loop-helix architecture similar to the EF-hand domain of PLC- δ 1 (Fig. 2).

Catalytic domain

The catalytic domain (residues 299–606) contains regions of highest sequence similarity, X and Y, among different mammalian phospholipases³. The topology of this domain resembles that of a distorted but closed TIM-barrel (Fig. 4a; nomenclature T β 1–T β 8 for β -strands, T α 1–T α 7 for α -helices). The X-region of PLC- δ 1 (residues 299–445) forms half of the TIM-barrel-like architecture with a typical $\beta\alpha\beta\alpha\beta$ -motif. The other half of the barrel is formed by the Y region (residues 489–606). Unlike a typical TIM-barrel, this half lacks a helix between T β 5 and T β 6. Instead, the connection between T β 5 and T β 6 is accomplished by an extensive loop region (residues 502–517) that appears to be rather flexible, as indicated by missing density for residues 511–513 and otherwise high temperature factors. The rest of the Y region folds up in $\beta\alpha$ repeat units, as observed in other TIM-barrels. The region between X and Y (residues 446–488) is not visible in the electron density. This region consists of a stretch of charged residues that appears to be dispensable for enzyme activity²⁰. In this region, the

γ -isozymes have a major regulatory element consisting of a split PH domain, one SH3 and two SH2 domains.

The active site is located on the C-terminal end of the eight-stranded β -barrel forming a rather broad ellipsoidal depression with dimensions of approximately $18 \times 13 \text{ \AA}$ at its outer rim and with a maximal depth of $10 \text{ \AA}$. The catalytic residues His 311 and His 356 (refs 21–23) project their side chains into the active-site depression, but are part of two distinct structural features of the TIM-barrel. His 311 is positioned on the tip of a β -bulge (residues 310–313). His 356 is located at the beginning of a hairpin loop (residues 356–361). This loop is part of a small, mixed β -sheet containing the C-terminal ends of β 2 and β 3 (residues 343–345, 390–392) and a region connecting β 2 with α 2 (residues 353–355, 362–364).

Like the catalytic domain of the mammalian enzyme, the structure of a bacterial PI-PLC from *Bacillus cereus* has a similar, distorted TIM-barrel topology²⁴, although there is little sequence identity in the X region²¹ and insignificant similarity in the Y region.

C2 domain

C2 domains are protein modules of about 120 residues identified in single or multiple copies in more than 40 proteins, many of which are involved in signal transduction and membrane interactions²⁵. The structure of only one C2 domain, an isolated C2 domain from synaptotagmin I, has been determined²⁵. The C2 domain of PLC- δ 1 (residues 626–756) forms an eight-stranded antiparallel β -sandwich (Fig. 3b; nomenclature for β -strands, C β 1–C β 8). A comparison with the structure of the synaptotagmin C2 domain shows that the two domains have a very similar structure but different topologies (Fig. 3b). Starting from the topology of the PI-PLC C2 domain, one could arrive at the synaptotagmin topology by joining C β 1 and C β 8 of the PI-PLC C2 domain, and cutting between C β 7 and C β 8 (Fig. 3b). Nevertheless, both β -sandwiches are structurally very similar with an overall r.m.s. deviation of $1.4 \text{ \AA}$ for 109 equivalent α -carbons.

The structure of the synaptotagmin C2 domain²⁵ shows that four

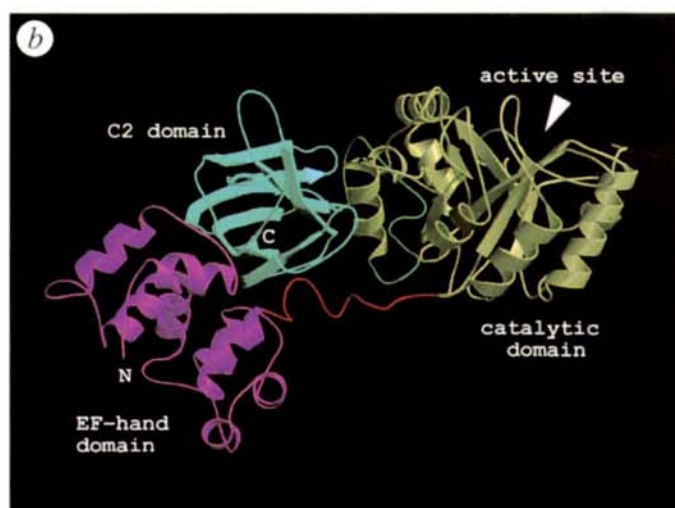
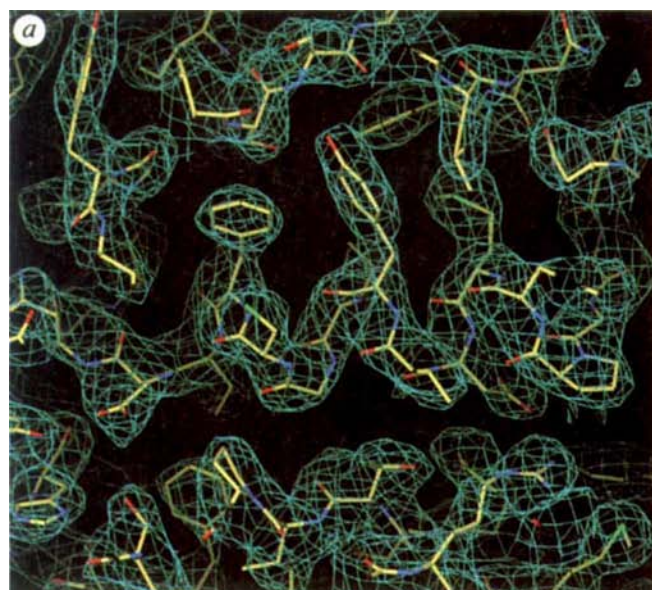


FIG. 1 Three-dimensional structure of PLC- δ 1. *a*, A region from a representative electron-density map calculated at $3.0\text{-\AA}$ resolution using solvent-flattened MIR phases. The structural model corresponds to the refined structure of the enzyme. The region shown is part of the interface between the catalytic domain and the C2 domain. *b*, Ribbon diagram representation of the PLC- δ 1 molecule. The catalytic domain is shown in yellow, the N-

terminal EF-hand domain in purple, and the C-terminal C2 domain in cyan. The linker between the catalytic and EF-hand domain is red, the linker between the catalytic and C2 domain is green. The C2 domain makes extensive contacts with both the EF-hand domain ($1,617 \text{ \AA}^2$ buried in the interface) and the catalytic domain ($1,089 \text{ \AA}^2$). This figure was prepared with the program O⁴³ (in *a*), and MOLSCRIPT⁴⁶ and RASTER3D⁴⁷ (in *b*).

aspartate residues, equivalent to Asn 645, Asp 653, Asp 706 and Asp 708 in PLC- δ 1, loosely coordinate a calcium ion. These residues, suggested to be the calcium-mediated lipid-binding site of synaptotagmin, belong to two loops that appear to be highly flexible in both synaptotagmin and PLC- δ 1. For an unambiguous crystallographic identification of putative calcium-binding sites in such a mobile region, we examined crystals soaked with the calcium analogue lanthanum. Two adjacent binding sites were revealed around Ser 650 to Asp 653, and Asp 706 to Asp 708 (Fig. 3b). In the membrane-binding protein annexin V, the recognition site for the head group of phosphatidylserine consists of two adjacent calcium sites²⁶. A similar mode of phospholipid binding by the PI-PLC C2 domain might be possible.

Stereospecific InsP_3 binding

The partly buried InsP_3 molecule in the active-site cleft gives a concise model for the interaction of PLC- δ 1 with the head group of the substrate PtdInsP_2 . With the exception of the 6-hydroxyl group, all other chemical groups of the InsP_3 molecule are

stereospecifically recognized by an extensive hydrogen-bonding network and specific charge-charge interactions (Fig. 4c). Binding of InsP_3 and calcium is not accompanied by significant structural changes.

The aromatic ring of Tyr 551 is almost parallel with the sugar ring of InsP_3 and forms numerous van der Waals interactions with it. Similar interactions are found in other protein/carbohydrate complexes²⁷. A 6-coordinated calcium ion is found at the bottom of the active site, consistent with the strict calcium requirement for PI-PLC activity³. A prominent feature is its ligation to the axial 2-hydroxyl group of InsP_3 (Fig. 4b). The remainder of the calcium ligands are the highly conserved residues Asn 312, Glu 341, Asp 343 and Glu 390. Consistent with this, the mutation of Glu 341 to Gly in human PLC- δ 1 (ref. 21) destroys enzyme activity. The importance of the 2-hydroxyl group of the substrate is substantiated by the observation that 2-deoxy- PtdIns is not hydrolysable by mammalian PI-PLCs²⁸ and exhibits only weak binding²⁹. The 1-phosphoryl group of InsP_3 forms hydrogen bonds to the NE2 atoms of His 311 and His 356, residues that are essential for

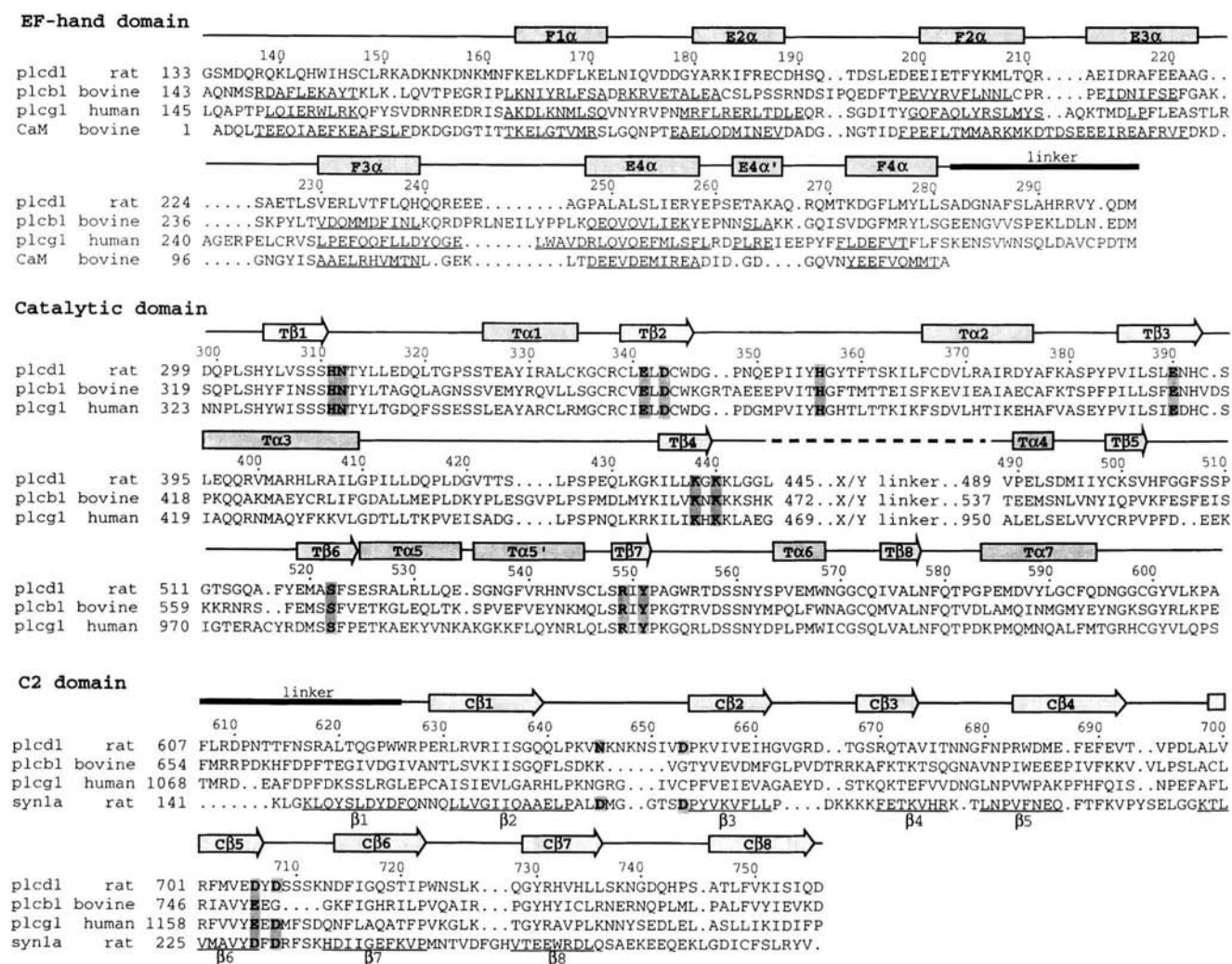


FIG. 2 Secondary structure elements of the rat PLC- δ 1 sequence and alignment with a set of related sequences. The sequence alignments for the PI-PLCs were produced with the program CLUSTAL W⁴⁸ and adjusted manually. PLC- β and PLC- γ are represented by β 1 and γ 1 isozymes. The sequence similarity among PI-PLC isozymes has been found throughout selected regions³. For the β 1 and γ 1 isozymes, helical elements in the EF-hand domain as predicted by the program PHD⁴⁹ are underlined. The EF-

hand and C2 domains of PLC- δ 1 were aligned with bovine calmodulin¹⁸ (CaM) and synaptotagmin I²⁵ (syn1a) on the basis of their structures. Reported secondary structure elements in calmodulin and synaptotagmin I are underlined. Residues important for the binding of InsP_3 and calcium in the active site as well as putative calcium ligands in the C2 domain are highlighted.

enzyme activity^{21–23}. Because the equatorial 3-hydroxyl group of InsP_3 is engaged in hydrogen bonds with Glu 341 and Arg 549 at the bottom of the active site, there is insufficient space for phosphoinositides phosphorylated at this 3-hydroxyl group. Indeed, 3-phosphoinositides are not substrates for mammalian PI-PLCs³⁰. Various salt bridges to the 4- and 5-phosphoryl groups are responsible for the observed substrate preference of $\text{PtdInsP}_2 > \text{PtdInsP} \gg \text{PtdIns}$ ¹⁰. The salt bridge between the 4-phosphoryl and the guanidinium group of Arg 549 appears to be one major determinant for substrate specificity, because mutation of this residue abolishes the hydrolysis of PtdInsP_2 , whereas hydrolysis of PtdIns remains unchanged²¹. Mutation of Lys 438, which forms the second salt bridge to the 4-phosphoryl group (Fig. 4c), also leads to loss of PtdInsP_2 hydrolysis³¹.

Mechanism of phosphoinositide hydrolysis

The structure of the $\text{InsP}_3/\text{Ca}^{2+}/\text{PLC-}\delta 1$ complex supports a sequential reaction scheme^{32–34} analogous to the action of ribonucleases such as A or T1 on RNA substrates. In a first step, a cyclic phosphodiester intermediate is formed by nucleophilic attack of the axial 2-hydroxyl group of PtdInsP_2 (ref. 34). In a second step, it undergoes hydrolysis to yield acyclic InsP_3 (Fig. 4d). The direct coordination of the 2-hydroxyl group to calcium might facilitate deprotonation by lowering the pKa of this group. For bacterial PI-PLCs, a histidine residue equivalent to His 311 was proposed to act as a general base in the deprotonation of the 2-hydroxyl group²⁴. In PLC- $\delta 1$, the imidazole group of His 311 appears to be too distant (3.6 Å) and unfavourably oriented to form a hydrogen bond with it. The only potential alternatives as a general base would be Glu 341 and Glu 390, which form hydrogen bonds to the 2-hydroxyl group. However, these residues would be less favourable proton acceptors in the close proximity to the calcium ion. Additional kinetic analyses of site-specific mutants will be necessary to identify conclusively the general base. His 356 is suitably positioned to act as a general-acid catalyst for the protonation of the diacylglycerol leaving group. The second step is the hydrolysis of the 1,2-cyclic-inositolphosphate intermediate, where His 356 promotes the nucleophilic attack of water by

general-base catalysis, and the general base of the first step becomes now the acid catalyst (Fig. 4d).

The PLC- $\delta 1$ structure suggest that the central role of calcium in catalysis is to act together with His 311 to stabilize the highly negatively charged pentavalent phosphoryl transition state. The bacterial enzyme appears to accomplish this stabilization with basic residues²⁴ instead of a calcium ion. Based on sequence alignments, residues equivalent to the calcium ligands Asp 343 and Glu 390 are substituted by arginine and lysine in the bacterial enzyme.

Substrate access to the active site

The membrane-resident nature of PI-PLC substrates requires that the catalytic domain adsorbs to the membrane so that its active site gains access to the scissile phosphodiester bond. The active-site depression itself has no hydrophobic surface features that might accommodate the lipid portion of phosphoinositides, but the rim surrounding the active site has a distinct hydrophobic ridge (Fig. 5a). This hydrophobic ridge is formed by three loops connecting T β 1 with T α 1, T β 2 with T α 2, and T β 7 with T α 6 (Fig. 5b). The structure of a complex of a CHAPSO detergent molecule with the enzyme suggests that this detergent might mimic the interaction of the catalytic domain with the lipid membrane. The hydrophilic moiety of CHAPSO extends into the active site so that its terminal sulphonyl group occupies a position similar to the 1-phosphoryl group of InsP_3 . Its steroid portion binds to the hydrophobic ridge (Fig. 5b), whereby it might simulate the hydrophobic groups of membrane lipids. This implies that the ridge might penetrate the hydrophobic portion of the membrane during interfacial catalysis to facilitate entry of phospholipid substrates into the active site. Based on the dependence of the enzyme activity and membrane surface pressure, it was estimated that about 1 nm² of the protein surface penetrates the membrane³⁵. This is roughly consistent with the area of the hydrophobic ridge.

Implications for membrane binding

PI-PLC contains two domains, PH and C2, that are thought to be

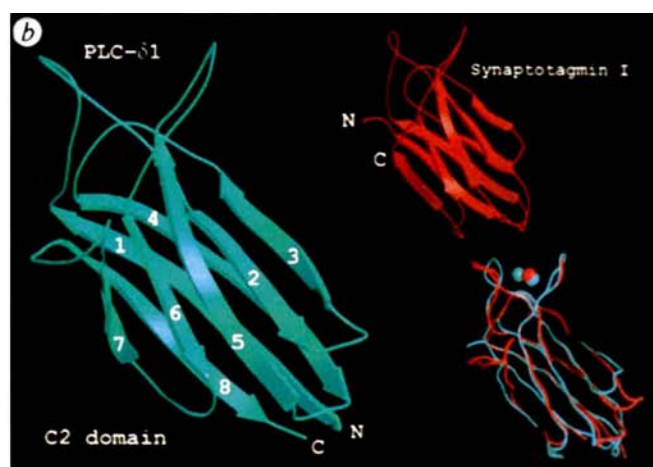


FIG. 3 Structures of the non-catalytic domains of PLC- $\delta 1$. The secondary structure elements are indicated. a, Ribbon diagrams for the EF-hand domain of PLC- $\delta 1$ (purple). Only the N terminus is marked. The inset in the top right shows a superposition of the backbone trace with bovine calmodulin (yellow). The N- and C-terminal lobes of calmodulin¹⁸ were individually superpositioned on the corresponding lobes of the PI-PLC EF-hand domain (1.3 Å r.m.s.d. for 40 C α atoms and 1.9 Å r.m.s.d. for 59 C α atoms). b, The C2 domain of PLC- $\delta 1$ (cyan) and comparison to the structure of the first C2 domain of synaptotagmin I²⁵ (red). Both domains superimpose with an r.m.s.d. of 1.4 Å for 109 structurally equivalent C α atoms.

The superposition also shows the locations of two lanthanum ions (cyan) in the C2 domain of PI-PLC and the calcium ion (red) as found in the synaptotagmin structure²⁵. The topological permutation between the two C2 domains leads to a different location of the N and C termini relative to the β -sandwich and the putative calcium/lipid-binding site. The lanthanum ions were located in lanthanum chloride soaked PI-PLC crystals as 14 σ and 12 σ peaks in difference Fourier maps. For comparison, a 15 σ peak for lanthanum is observed in the active site that is coincident with the position of the catalytic calcium in the $\text{InsP}_3/\text{Ca}^{2+}/\text{PLC-}\delta 1$ complex.

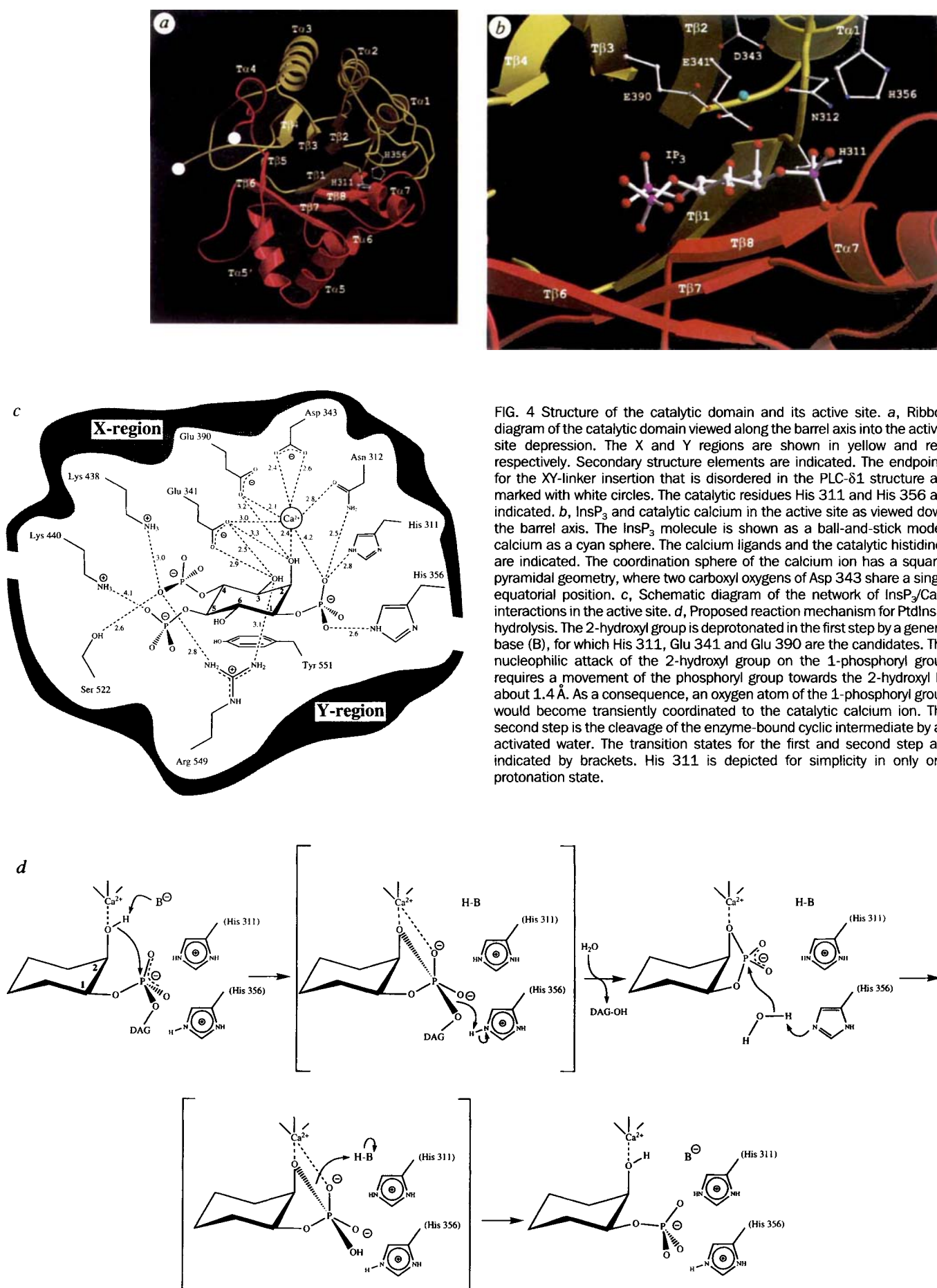
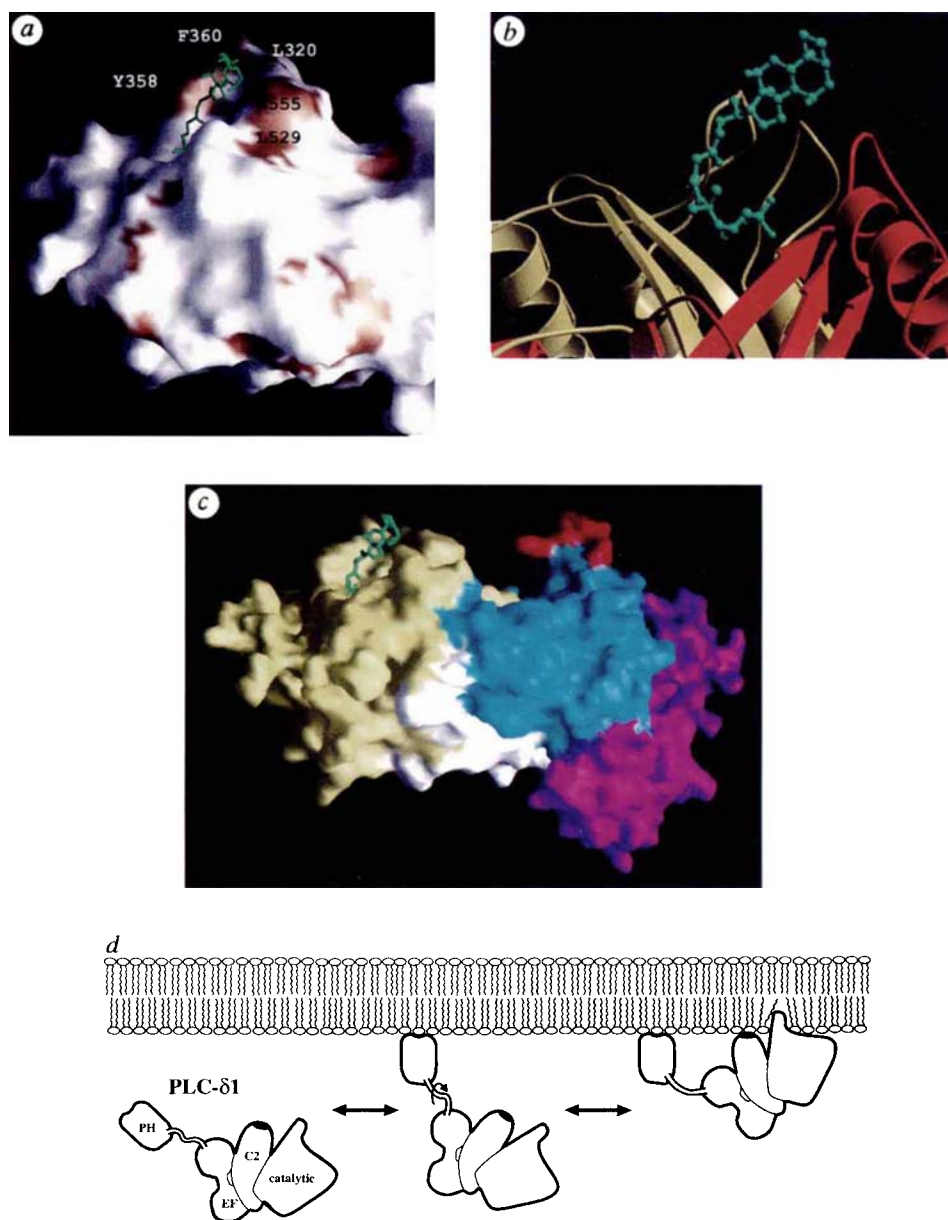


FIG. 5 Detergent interaction and 'tether-and-fix' model for reversible membrane interaction. **a**, Surface representation of PLC- δ 1 viewed approximately perpendicular to the barrel axis of the catalytic domain. Surface patches belonging to hydrophobic residues are shown in bronze, all other residues in white. An increased probe radius of 4 Å was used for the generation of the molecular surface to simulate accessibility to lipid groups. The bound CHAPSO detergent molecule is shown in green. **b**, Location of the CHAPSO detergent molecule in the active site. The conformation of the bound CHAPSO molecule (green) was deduced from a difference Fourier map at 2.4 Å. The X and Y regions are shown in yellow and red, respectively. **c**, Overall surface representation of the PLC- δ 1 molecule. The orientation chosen (same as in **a** and **b**) shows that the active-site opening, hydrophobic ridge and the putative calcium-mediated lipid-binding site are approximately in the same plane. The coplanar nature of these sites would allow them to interact simultaneously with the membrane. The colouring scheme follows Fig. 1b with the exception that the linker regions are shown in white. The putative calcium-mediated lipid-binding site in the C2 domain is in red. **d**, 'Tether-and-fix' model for reversible membrane association and regulation of PLC- δ 1. The putative calcium-mediated lipid-binding region of the C2 domain is shaded. GRASP⁵⁰ was used to generate **a** and **c**.



important for membrane binding in many proteins. We are proposing a mechanism involving two steps, tether and fix, for the interaction of PLC- δ 1 with membranes; each step could be important for regulation of PI-PLC isozymes (Fig. 5d). The PH domain of PLC- δ tethers the enzyme to the membrane by specific binding to PtdInsP₂, and the C2 domain fixes the catalytic domain in a productive orientation relative to the membrane. The PH domain is required for processive, but not non-processive, catalysis³⁶. In addition to its role in anchoring the enzyme to the plasma membrane³⁷, the PH domain has been suggested to have a regulatory role by binding to the soluble reaction product InsP₃ (ref. 12). There is likely to be a flexible connection between the PH domain and the rest of the enzyme, which might explain the high disorder we observe for EF-hands 1 and 2. In a recent characterization of isomorphous crystals of full-length PLC- δ 1 (data not shown), there is a similar degree of disorder for these EF-hands and a complete absence of density assignable to the PH domain. In contrast, the C2 domain has an extensive and rigid interface with the catalytic domain. Furthermore, our structure reveals the putative calcium-dependent lipid-binding site in the C2 domain on the same side of the enzyme as the active site and the

hydrophobic ridge (Fig. 5c). These observations are in accord with the proposed role of the C2 domain in forcing the catalytic domain into a fixed, productive orientation on the membrane. Moreover, this could provide a site for regulation by calcium and membrane phospholipid composition. The essential role of the C2 domain in PLC- δ 1 is underlined by the finding that deleting it leads to loss of enzyme activity with membrane substrates^{20,38}. PLC- β and PLC- γ have specific regions of interaction with their upstream regulatory components which may include other tethering/fixation points.

Conclusion

The PLC- δ 1 structure provides a framework for understanding how the mammalian PI-PLC superfamily generates second messengers. Furthermore, we believe this to be the first description of either an EF-hand domain or a C2 domain in the context of a multidomain protein. This structure might also serve as a representative of a large class of proteins involved in signal transduction that use a modular architecture for reversible membrane association, such as protein kinase C, cytosolic phospholipase A2, or phosphatidylinositol 3-kinase.

The observation that all active-site residues involved in the binding of both the substrate and the catalytic calcium are strictly conserved in eukaryotic PI-PLC isozymes implies an early evolutionary connection between PtdInsP₂ hydrolysis and calcium regulation. Substrate-mediated tethering by the PH domain,

additional calcium-binding sites in the C2 domain, and possibly also in some of the EF-hands, indicate a sophisticated regulatory pattern for PLC- δ 1 activity. Clearly, more studies on isolated components of this system are required before the contribution of each of them can be completely understood. □

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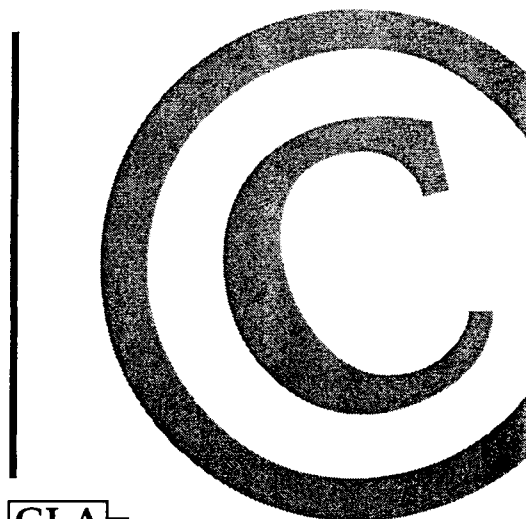
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How filaments of galaxies are woven into the cosmic web

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LARGE-SCALE structure in the distribution of galaxies is thought to have evolved through gravitational instabilities from small density fluctuations in the (largely homogeneous) early Universe. This structure of galaxies consists of rich and poor clusters, connected by filaments and sheets, with regions largely devoid of galaxies (voids) in between¹. Numerical simulations of the growth of initial density fluctuations through a nonlinear regime, motivated by the likely physics of the early Universe, also show a network of filaments and voids^{2,3,18}, but the origin of this picture of filaments as the dominant structure was not well understood. Here we show that the ‘web’ of filaments that defines the final state in these simulations is present in the initial density fluctuations; the pattern of the web is defined largely by the rare density peaks in the initial fluctuations, with the subsequent nonlinear evolution of the structure bringing the filamentary network into sharper relief. Applying these results to the observed galaxy distribution, we suggest that ‘superclusters’ are filamentary cluster–cluster bridges, and we predict that the most pronounced filaments will be found between clusters of galaxies that are aligned with each other and close together.

Two classical theories have largely defined how people have thought about structure in the Universe, the (Russian) pancaking picture^{4,5} and the (Western) hierarchical clustering picture⁶. The Zel’dovich theory⁴ has been applied to the models with fluctuation power on cluster scales but none on galactic scales. It led to a highly influential description of the medium: planar pancakes form first (as fold caustics), these drain into filaments which in turn drain into clusters, with the entirety forming a cellular network of sheets. The rival hierarchical clustering theory has been used in models with initial density fluctuation power decreasing with increasing scale, with a characteristic mass scale for nonlinear objects increasing with time^{6,7}. The main idea used here was a merging-haloes picture, where haloes are associated with peaks in the ‘background’ linear (gaussian-distributed) density field⁸, that is, of the rare events in the medium.

A sample ‘cosmic web’ N -body pattern is shown in Fig. 1 top panel, for group and cluster formation in a ‘cold dark matter’ model universe: as the density threshold drops from high values the regions which first emerge are clusters, then arms stretching from the clusters, which ultimately join to form the predominantly filamentary network. The first pattern to percolate is filamentary and it is that which the eye picks out. This basic pattern is seen on smaller scales at higher redshift in simulations, with bright galaxies playing the role of clusters.

The nonlinear dynamical evolution of an initially cold medium is described by a map, $\mathbf{x}(\mathbf{r}, t) = \mathbf{r} - \mathbf{s}(\mathbf{r}, t)$, from initial state (lagrangian) space $\mathbf{r}$ to final state (eulerian) space $\mathbf{x}$. Lagrangian coordinates specify the initial positions of particles, Eulerian ones specify their position at an arbitrary moment. In the early linear phase, the displacement field $\mathbf{s}$ is small and the map is one-to-one, but eventually when particle orbits cross the map becomes multi-valued. We show here that the filamentary web is a consequence of the distribution and spatial coherence of the strain field in the medium. To establish this, we shall split the displacement field $\mathbf{s} = \mathbf{s}_b + \mathbf{s}_f$ into a background field $\mathbf{s}_b$ smoothed over a large scale R_b and a fluctuating field $\mathbf{s}_f$ built from shorter-wavelength com-

ponents. The conceptual picture is one of a smooth stately large-scale single-stream flow $\mathbf{s}_b$ on which is superposed a relatively chaotic nonlinear short-distance flow $\mathbf{s}_f$ in which orbit crossings abound^{9,10}. We characterize the filtering by the linear r.m.s. density fluctuation amplitude smoothed on scale R_b , $\sigma_\rho(R_b, t)$, which we must restrict to be $\lesssim 1$ for this picture to be valid. The linear approximation (in lagrangian space), $\mathbf{s}_b(\mathbf{r}, t) = D(t)\mathbf{s}_b(\mathbf{r})$, (where $D(t)$ describes the linear growing mode of fluctuations), defines the Zel’dovich map, which by itself gives a reasonable description of the overall pattern of N -body simulations.

The ‘background’ strain field is defined by $e_{bij}(\mathbf{r}) \equiv -(\nabla_i s_{bj} + \nabla_j s_{bi})/2$. In linear theory, the shear tensor is just its time derivative, $\dot{e}_{bij}$, $\mathbf{s}_b$ is proportional to the gradient of the smoothed gravitational potential and hence the smoothed peculiar tidal field acting on a patch of matter is proportional to e_{bij} . The strain tensor has eigenvalues $-\lambda_{vj}$, which we order by $\lambda_{v3} \geq \lambda_{v2} \geq \lambda_{v1}$. We also express⁸ the λ_{vj} in terms of the height of the density fluctuation $v_b = (\lambda_{v3} + \lambda_{v2} + \lambda_{v1})/\sigma_\rho$, the shear ellipticity $e_v = (\lambda_{v3} - \lambda_{v1})/(2v_b\sigma_\rho)$ and prolativity $p_v = (\lambda_{v1} + \lambda_{v3} - 2\lambda_{v2})/(2v_b\sigma_\rho)$; thus $e_v \geq 0$ and $-e_v \leq p_v \leq e_v$. In this notation, extreme sheet-like structures would have $p_v \approx -e_v$, extreme filaments would have $p_v \approx e_v$. Both are statistically unlikely: in the conditional distribution⁹ $P(\{\lambda_{vj}\} | v_b)$, $\langle e_v | v_b \rangle \approx 0.54v_b^{-1}$ is significantly nonzero while $\langle p_v | v_b \rangle$ is zero (and the dispersion is $\sim 0.2v_b^{-1}$ for both); and we find¹¹ regions with $p_v \approx 0$ and large e_v favour filaments over sheets. The Zel’dovich-mapped conditional distributions are qualitatively similar¹¹ and further favour (ribbon-like) filaments.

Figure 1, middle right panel, shows the structure of the isodensity surface of the Zel’dovich background field map at the percolation threshold. Even with this approximate dynamics, the web defined by filaments that was evident in the N -body simulation is clearly seen: sheets are not the dominant elements. With full nonlinear rather than Zel’dovich dynamics, the cluster regions occupy less eulerian volume which leads to an even more enhanced filamentary character. The prominent filaments in the final state are there in fattened form in the initial state, as Fig. 1 also shows.

To probe quantitatively the coherent structures in the initial conditions, we turn to a new language: conditional multiple-point correlation functions in lagrangian space—that is, statistically averaged density and displacement fields subject to various constraints on the shear at multiple points. Consider first the behaviour of an ambient patch about a single random point in the medium at which we specify the shear parameters e_v and p_v to be the typical ones corresponding to a given height v_b in the lagrangian space: we find that the typical density contours are more filamentary than sheet-like even before Zel’dovich mapping and the density coherence scale along the 1-direction, which is the filamentary axis, is greatly enhanced.

The remarkable size of the filaments is not, however, derivable from constraints given at a single point. For this, we turn to correlations constrained by at least two rare peak-patches in the medium. Examples are shown in Fig. 3. The peak-patch theory⁹ shows that it is statistically likely that, given a specific orientation of the shear tensor for a peak-patch, neighbouring peak-patches will be preferentially along the long 1-axis and have shear tensors aligned with it¹². A filament-like enhancement of overdensity bridges the aligned peaks. The reason for this phenomenon is that the high degree of constructive interference of the density waves required to make the rare peak-patches, and to preferentially orient them along the 1-axis, leads to a slower decoherence along the 1-axis than along the others¹³, and thus a higher density. When we vary the peak separation or the shear orientation, we see that the strong correlation bridge between two clusters gradually weakens as the separation increases, or as the shear tensor orientations become misaligned.

So we suggest the following theory of how clusters and their

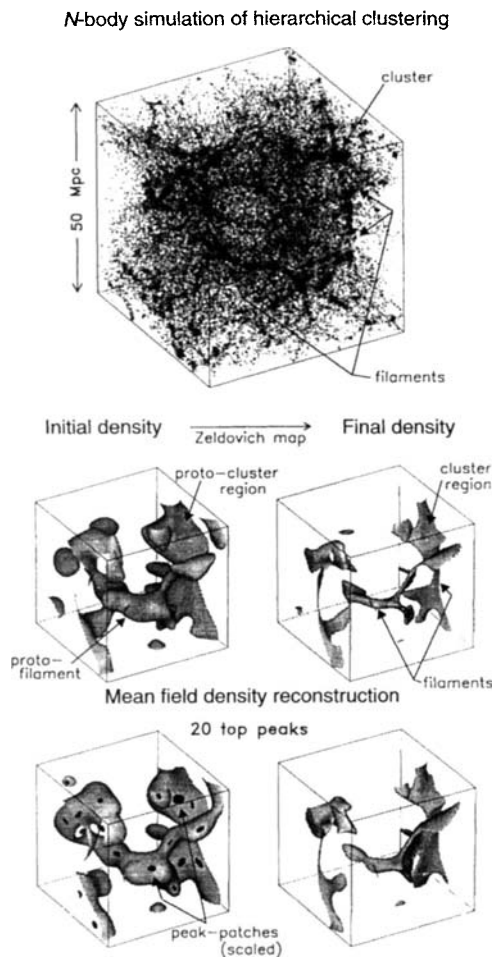


FIG. 1 Top panel, N -body simulation of a cold dark matter (CDM) model with 128^3 particles in a co-moving volume of $(50 h^{-1} \text{ Mpc})^3$ with periodic boundary conditions evolved to the r.m.s. density fluctuation amplitude $\sigma_8 = 0.7$ (where $\sigma_8 = \sigma_p$ at a top-hat filtering scale $R_b = 8 h^{-1} \text{ Mpc}$; h parametrizes the Hubble constant, $H_0 = 100 h \text{ km s}^{-1} \text{ Mpc}^{-1}$). One in ten particles are plotted. The pattern of connected filaments joining clusters evident here is quite general for viable hierarchical theories that explain the galaxy clustering data. The middle left panel shows the initial linear CDM overdensity field $\delta_L(\mathbf{r})$ for this simulation, smoothed on a gaussian scale $R_b = 3.5 h^{-1} \text{ Mpc}$, giving $\sigma_p = 0.65$. The threshold chosen is $\delta_L = 1\sigma_p$ because $\delta_L(\mathbf{r})$ percolates at this level. The middle right panel shows $\delta_z(\mathbf{x}, t)$, the overdensity of the Zel'dovich map of the smoothed initial conditions, at a contour threshold $\delta_z = 2$, just above where percolation in the final state occurs. The final state filaments are also seen to be present in the initial state. The Zel'dovich map does not imply that pancakes are the dominant overdensity enhancement structures for $\sigma_p(R_b) \sim 1/2$. Lower left panel shows the linear density correlation function $\langle \delta_L(\mathbf{r}) | 20 \text{ pks} \rangle$ constrained by the 20 most massive peak-patches (protoclusters) identified in the initial conditions, and the lower right shows its Zel'dovich map. $\langle \delta_L | 20 \text{ pks} \rangle$ is the mean field reconstruction of δ_L given 'observations' of the values of the overdensity δ , the shear tensor e_{ij}^{δ} and the displacement $\mathbf{s}_b$ at N peak-patch points $\mathbf{r}_{pk}$ averaged over the peak-patch size R_{pk} , calculated by application of a well known theorem for a constrained gaussian field⁸, for $9N$ constraints. The extremum requirement of vanishing density gradient at $\mathbf{r}_{pk}$ adds a further $3N$ constraints, but is unimportant in practice. Although the positions are not counted explicitly as constraints, changing them radically alters the way the mean fields look. The greater N is, the smaller are the fluctuations about the mean. The patches are indicated by black ellipsoids of overall size proportional to R_{pk} and shape defined by the shear tensor orientation, with the shortest axis corresponding to the highest shear eigenvalue. We therefore identify the filaments as 'correlation bridges' between the collapsed peak-patches, forming a web, but no classical pancakes.

filamentary bridges weave a web. Clusters nearer than the mean cluster separation can have strong correlation bridges. There are many such pairs as clusters are statistically biased⁸. The bridge breaks if the separation is too large, isolating the clusters from each other—unless there is a cluster in between to which both

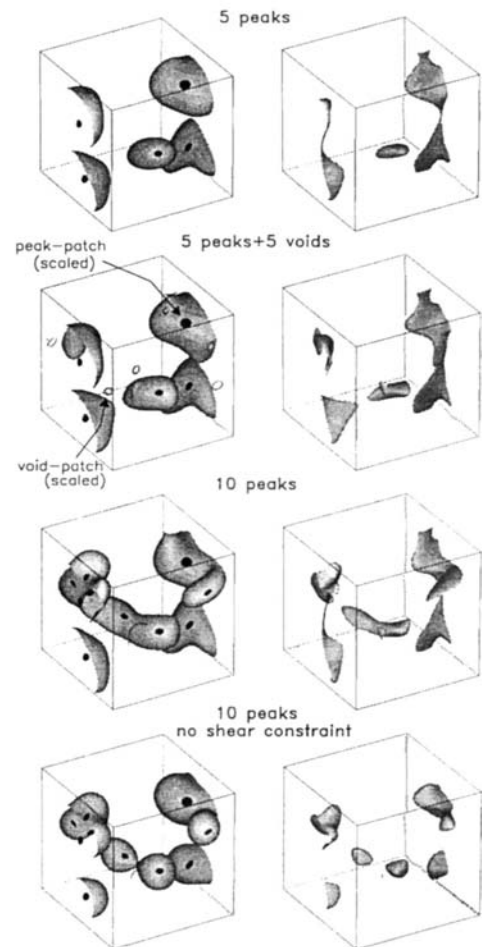


FIG. 2 In Fig. 1, the reconstruction $\langle \delta_L | 20 \text{ pks} \rangle$ in the lower panel compares well with the δ_L of the middle panel. We show here, in the top three panels, how the reconstruction fares with fewer of the most massive peak-patch and void-patch constraints imposed. The same thresholds were used as in Fig. 1. Some strong bridges seen in the 20 pks reconstruction, $\langle \delta_L | 20 \text{ pks} \rangle$, are not as evident in the $\langle \delta_L | 10 \text{ pks} \rangle$ -field, but emerge at lower thresholds. Using the top 20 peaks rather than N peaks and N voids (treated as 'inverse-peaks' and shown as open ellipsoids) is better if we are focusing only on regions of higher density contrast—which is observationally most relevant for most astrophysical observables. The situation would be reversed if we were concentrating on the voids. Note that the void-patch constraints create high mean field regions in between them, just where less rare peak-patches reside. Using voids is not as precise a way to get that structure for the same computational effort. The bottom panels, using constraints on the peak height and the velocity (or displacement field) only, shows how important the anisotropic shear constraints are for defining the structure.

METHODS. The peak-patch catalogue was constructed using the method of ref. 9. First, we find all peaks above a threshold ($\delta_{Lc} = 1.686$) in the linear density field smoothed on a hierarchy of (25) gaussian filter scales. Second, we calculate for each peak the radius R_{pk} at which the interior region is predicted to have just collapsed in all three dimensions at the redshift in question, using a homogeneous ellipsoid model for interior dynamics which includes both the interior tidal field and a linear-evolution approximation to the exterior tidal field acting on the patch. Last, we remove from the list smaller-scale peaks whose centres lie within larger-scale peaks, and rank-order the remaining peaks in mass. We find about 400 peak-patches in our $(50 h^{-1} \text{ Mpc})^3$ volume.

have extended their filamentary bridges. To make this into a network, consider laying down the rare events in the medium according to the clustering pattern of peak-patches which become clusters when they evolve dynamically. The correlation bridges arch from cluster to cluster in much of the domain, and these dynamically evolve to filaments, creating the network and containing the bulk of the mass. The typical scale of the segment (bridge) of the filamentary network is $\sim 30 h^{-1}$ Mpc. The order in which the physically significant structures arise is basically the inverse of that in the classical pancake picture: first, high-density peaks, then filaments between them, and possibly afterwards the walls, defined as the rest of the mass between voids.

The proof that this theory works is the ability to capture sequentially more and more large-scale structural features by imposing constraints from a list of peak-patches rank-ordered in

mass. It gives us a method for reconstructing initial states using the rarest peaks (clusters) and, through a background-field map, the main features of the final states. The excellent reconstruction shown in the lower panels of Fig. 1 develops as peaks are sequentially added, as is shown in Fig. 2. The anisotropic peak-strain constraint is essential for success in reconstruction.

The list of peak-patches (and void-patches) is therefore a powerful way to rank-order and maximally compress the information stored in the initial conditions, showing what is essential to define structures on the basis of a modest set of local measurements. The characterization is spatially localized: only the rare events nearby define the region. A modest extrapolation of the work reported here confirms the conjecture in ref. 9: within a cluster-scale peak-patch, the rarest group-scale peak-patches defined at moderate redshift will define the dominant evolutionary characteristics (the merger history), and one has to work one's way down the group-patch list only if one wants finer and finer dynamical detail.

The web pattern depends upon the shape of the initial-state (linear) power spectrum, $\langle |\delta_L(k)|^2 \rangle$, which is usually parametrized by a local power law index $n_{p,eff}(k) = d \ln \langle |\delta_L(k)|^2 \rangle / d \ln k$. In our figures, we used the standard cold dark matter (CDM) spectrum, which has $n_{p,eff} \approx -1.2$ on cluster scales. When we steepen the spectrum to $n_{p,eff} \geq 0$, the clusters become less clustered and the coherence of the web is lost, and although some filaments remain they are weaker and shorter. When we flatten it to $n_{p,eff} \lesssim -2$, the clusters are more clustered, the coherence is more pronounced and the filaments are both strengthened and widened (ribbons). We also see stronger membranes in the regions between the filaments when a number of clusters are close together. These are sheet-like structures, but are not classical pancakes.

There are several immediate applications of our theory to observations. We suggest that superclusters are predominantly cluster-cluster bridges; no image from the cosmology of the 1980s was as powerful as the Center for Astrophysics picture¹⁴ of the Coma cluster and its environs, with a great arm of galaxies spanning Coma and Abell 1367. 'Great Wall' features would be interpreted as the membrane-like webbing between the filaments, and would be more pronounced the flatter the spectrum; for example, theories which match cluster observations have $n_{p,eff} \geq -1.6$ on cluster scales, hence on average would be more membrane-like than structures in the CDM-dominated Universe would be.

We predict that the strongest filaments exist between highly clustered and aligned clusters. This should be especially notable in the mass distribution around systems of clusters reconstructed with weak gravitational lensing¹⁵, which uses the projected strain (shear) field. It also may affect the strategy of the search for X-ray-emitting gas¹⁶ or the Sunyaev-Zel'dovich effect in filaments. Most theories predict density fluctuation spectra are flatter at galactic than cluster scales, with $n_{p,eff} \lesssim -2$ appropriate for redshifts $z \geq 3$. The neutral hydrogen absorption by the filaments existing then would be observed as part of the quasar Lyman- α forest¹⁷, with column densities $N_{HI} \lesssim 10^{14.5} \text{ cm}^{-2}$ and a characteristic (co-moving) filamentary size roughly the distance between dwarf galaxies, $\sim 1 h^{-1}$ Mpc. We also expect to find filaments of dwarf galaxies between normal galaxies at similar redshifts. $\square$

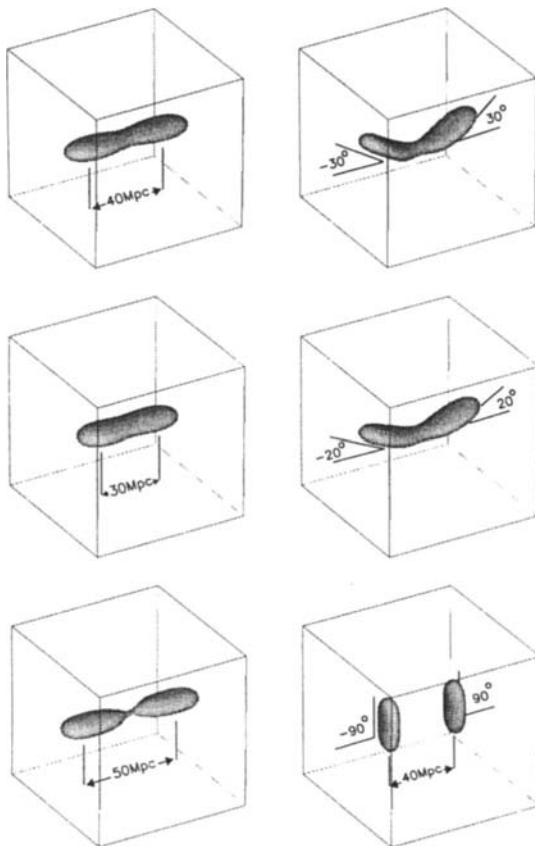


FIG. 3 Zel'dovich-mapped correlation functions constrained by shear at two peaks show the effect of changing separation and shear-tensor orientation on the strength of the correlation bridge. The bridge between two clusters gradually weakens as the separation increases, breaking by $50 h^{-1}$ Mpc, or as the shear tensor orientations become misaligned. For the left-hand panels, shear tensors are aligned and the peak separations are as shown. Mean-shear parameters appropriate for Abell-cluster peak-patches were adopted: $v = 3$, $e_v = 0.18$ and $p_v = 0$. (The average separation of clusters of this richness is $\sim 50 h^{-1}$ Mpc and their correlation length is $\sim 20 h^{-1}$ Mpc; that is, clusters come in patches¹³.) To avoid too much interior dynamics in the patches, the Zel'dovich map was used with gaussian smoothing scale $R_0 = 5 h^{-1}$ Mpc ($\sigma_p = 0.54$), the same as for the peaks (whose top-hat scale is $R_{pk} \approx 10 h^{-1}$ Mpc). The contour shown is at $\delta_{Z_0} = 1$, about where percolation occurs in the N -body computation of Fig. 1 at this σ_p smoothing. Note that in lagrangian space the length of the filament bridging the two peak regions is similar to the diameter of the peak patches. For the right-hand panels, the separation is fixed at $40 h^{-1}$ Mpc and the misalignment angles relative to the line joining the peaks are: $\pm 30^\circ$ (top), $\pm 20^\circ$ (middle) and $\pm 90^\circ$ (bottom), when the bridge is fully broken. At this $\delta_{Z_0} = 1$ level, the bridge is also broken with $\pm 45^\circ$ orientation, but joins at a lower threshold.

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Orbital migration of the planetary companion of 51 Pegasi to its present location

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THE recent discovery¹ and confirmation² of a possible planetary companion orbiting the solar-type star 51 Pegasi represent a breakthrough in the search for extrasolar planetary systems. Analysis of systematic variations in the velocity of the star indicate that the mass of the companion is approximately that of Jupiter, and that it is travelling in a nearly circular orbit at a distance from the star of 0.05 AU (about seven stellar radii). Here we show that, if the companion is indeed a gas-giant planet, it is extremely unlikely to have formed at its present location. We suggest instead that the planet probably formed by gradual accretion of solids and capture of gas at a much larger distance from the star (~ 5 AU), and that it subsequently migrated inwards through interactions with the remnants of the circumstellar disk. The planet's migration may have stopped in its present orbit as a result of tidal interactions with the star, or through truncation of the inner circumstellar disk by the stellar magnetosphere.

The first argument against the *in situ* formation of a planetary companion is based on models of the nebular disks³ that are known to exist around young stars⁴. The standard picture of the formation of a giant planet involves the coagulation and accretion of small particles of ice and rock in the disk⁵ until a core of about 15 Earth masses is built up; then gas, composed mainly of H and He, is accreted from the disk⁶. Standard disk models show that at 0.05 AU the temperature is about 2,000 K, too hot for the existence of any small solid particles. An alternative formation model⁷ involves a massive disk, whose self-gravity is comparable to that of the central object, in which a gaseous subcondensation could form by contraction under its own gravity. But recent detailed calculations of such massive disks⁸ indicate that they tend to form spiral arms and to transfer mass into the central star instead of fragmenting into subcondensations.

A second problem with the formation of a planet at 0.05 AU is that although the present evaporation rate of the planet is negligible, this effect would have been of major importance in the past. At 0.05 AU, the companion's effective temperature, due to stellar irradiation, is $\sim 1,300$ K. In order to determine the planetary radius R_p in the presence of such heating, we calculated the evolution of objects in the mass range $M_p = 1\text{--}10 M_J$ (jovian masses) using a standard stellar structure code^{9,10} with a non-ideal interior equation of state¹¹. The rotation of the planet is almost certainly tidally locked¹² so that the same hemisphere always faces 51 Peg. We assume that atmospheric motions and convection in

the interior redistribute the heat so that the dark side and the bright side have nearly the same temperature.

In Fig. 1 we show the evolution of R_p for various M_p . At 8 Gyr, the estimated age of 51 Peg, $R_p = 8.3 \times 10^7$ m for $1 M_J$, not much larger than the present radius of Jupiter (7.0×10^7 m). For these values, the escape velocity of a hydrogen atom is 12 times larger than the mean thermal speed, and a simple calculation of the Jeans escape rate¹³ shows that evaporation is negligible. A further process to be considered is hydrodynamic escape¹⁴ in which ultraviolet and X-ray radiation from the star are absorbed by hydrogen atoms in the planetary atmosphere and drive a planetary wind. A rough estimate, based on the observed X-ray flux of young stars¹⁴, shows that the effect of this process is also negligible. Thus the planet at present is quite safe against evaporation. The evaporation of a low-mass star or brown dwarf, another proposed explanation¹⁵ for the existence of the companion, would be even more difficult because the object would have a much higher surface gravity. But during the early history of a planet⁶ its radius is a factor of ten or more larger than the present radius, so the escape speed becomes much less and both evaporation mechanisms, along with ablation by the stellar wind, will prevent formation.

We propose that the companion was formed several AU away from the star through the standard process. Recent detailed calculations¹⁶ for the accretion of Jupiter at 5 AU have shown that it is possible for that planet to build well before the nebula dissipates. The protoplanet interacts tidally with the disk during its growth¹⁷. Let ν be the disk viscosity, M_* the stellar mass, ω the orbital frequency, and r_n the distance from the star at which the planet formed. If $M_p \gtrsim 40\nu M_*/(\omega(r_n)r_n^2)$ when its tidal radius, $(M_p/3M_*)^{1/3}r_n$, exceeds H (the vertical scale height of the disk), the protoplanet induces the formation of a gap^{18,19} in the disk near r_n so that growth of the planet stops. Standard disk models³ give $H(r) \approx 0.1r$, where r is the distance from the star. The disk evolves viscously on a timescale $\tau_v \approx r_n^2/\nu$ which is inferred to be $\sim 5 \times 10^6$ yr from infrared observations²⁰. The effective radius r_d which contains most of the disk mass observed in the infrared is⁴ ~ 100 AU. Applying these estimates to the gap formation conditions, we find $M_p \approx M_J$.

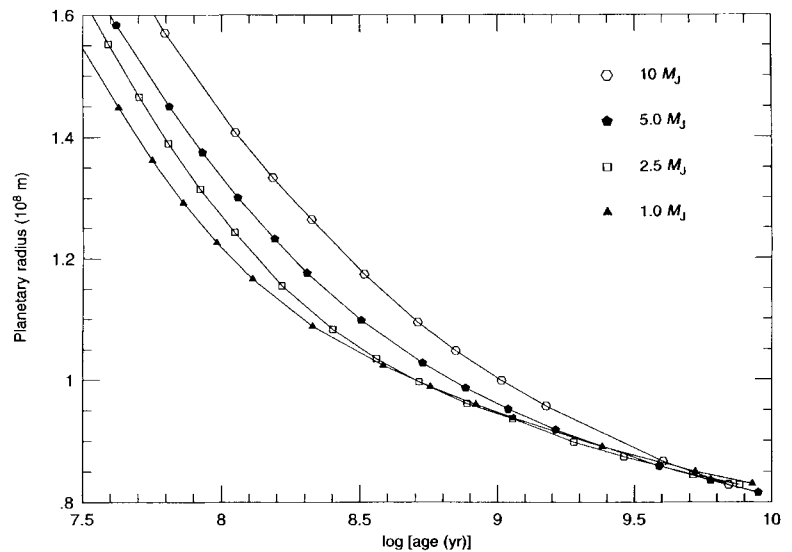
After the gap formation, angular momentum transfer continues and the protoplanet undergoes orbital migration coupled to the viscous evolution of the disk^{21,22}. The orbital radius of the planet (r_p) and that of the gap (both are still embedded in the disk) decrease on the timescale^{22,23} of τ_v . The planet essentially follows the material of the inner disk as it evolves towards the star. We now propose two possible mechanisms which suggest that this migration can terminate at ~ 0.05 AU and that the planet will not plunge into 51 Peg.

Mechanism 1. As the planet approaches 51 Peg, tidal friction can induce angular momentum exchange between the planet's orbital motion and the spin of the star. If R_* is the stellar radius and P the orbital period of the planet, the timescale for tidal evolution is¹²

$$\tau_t = r_p \left(\frac{dr_p}{dt} \right)^{-1} = \frac{P}{9\pi} \left(\frac{r_p}{R_*} \right)^5 \left(\frac{M_*}{M_p} \right) Q, \quad (1)$$

We estimate the dissipation parameter $Q_* = 1.5 \times 10^5$ for a main sequence star based on the observation²⁴ that the orbits of short-period pre-main-sequence binary stars and the main-sequence binary stars in the Pleiades cluster are circularized for $P \lesssim 5$ and 7 days, respectively. As young stars rotate more rapidly than their main-sequence counterparts²⁵, we assume that 51 Peg was rotating rapidly enough that the co-rotation point τ_{CR} (the distance from the star where an orbiting object has the same angular frequency as the stellar rotation) was inside 0.05 AU. The tidal effect then results in outward migration of the planet. Thus there may exist a radius r_c where the protoplanet's radial migration was halted by a balance between the inward push on it by the disk and the outward push from 51 Peg. At that point, the angular momentum transfer

FIG. 1 Planetary radius as a function of log age for objects (bottom trace to top trace) of masses 1, 2.5, 5 and $10 M_J$. The calculation assumes that the planet migrated to its present position during its first ten million years of existence. The plot shows the subsequent evolution, during which the orbit of the planet was stationary and it was heated by a central star whose luminosity was constant in time. For this evolutionary history, evaporation is not important.



equilibrium throughout the disk¹⁹ implies $\tau_v \approx \tau_r$ such that $r_c \equiv (9\pi\tau_r M_p / P_* Q_* M_*)^{2/13} R_*$, where P_* is the keplerian orbital period at R_* . Based on an estimate²⁶ of $R_* = 4R_\odot$ (where $R_\odot$ is the solar radius) during its early history, we find that this equilibrium can be established at 0.05 AU during the early epoch of 51 Peg.

But this equilibrium is only temporary. The disk material between the planet and the star will accrete onto the latter, leaving the planet with the remaining disk outside its orbit. The disk's surface density adjusts until a quasi-equilibrium state is attained in which the angular momentum flux is approximately constant with distance from the star. At this stage the planet's equilibrium radius is determined by the condition that the star's tidal torque on it, $M_p r_p^2 \omega_p / \tau_r$, is balanced by the angular momentum flux through the disk, $\sim M_d r_d^2 \omega_d / \tau_v$, where ω_d is a mean angular frequency of the disk and M_d is its mass. For $R_* = 4R_\odot$ we find that $r_c \approx 0.03(M_p/M_d)^{1/6}(\tau_v/5 \times 10^6 \text{ yr})^{1/6}$ AU. If the disk then dissipates sufficiently so that its mass $M_d \approx M_p$ and its evolution timescale lengthens so that $\tau_v \approx 10^8$ yr, then r_c could be close to the present orbital position of the planet. The dissipation must occur before the star contracts substantially ($<10^7$ yr) or spins down ($>10^8$ yr). In view of the rather precise timing and the relatively large R_* needed for this mechanism to work, we consider an alternative, as follows.

Mechanism 2. The spin periods of classical T Tauri stars are clustered²⁷ around 8 days, longer than those of the weak line T Tauri stars. One explanation for the 8-day periods is that the spin rate is controlled by coupling between the stellar magnetosphere and the disk²⁸. The presence of the magnetosphere would also clear²⁹ the inner disk out to a point slightly less than r_{CR} (0.08 AU for an 8-day period). Once the planet has spiralled in to $r_p = 0.05$ AU, angular momentum exchange between it and the disk occurs only via the 2:1 resonance at a reduced (by $\sim M_p/M_*$) rate^{17,30}. Because $r_p < r_{CR}$, the stellar tidal effect also continues to induce an inward migration. But as long as $R_* < 3R_\odot$, consistent with evolutionary tracks²⁶, τ_r is larger than the stellar contraction timescale, and the migration effectively stops near 0.05 AU.

After this time, in either case, τ_r and τ_v increase rapidly because the star contracts on a relatively short timescale, and the disk dissipates. During its contraction to the main sequence, 51 Peg may have spun up, if it conserved angular momentum, but once it reached the main sequence, the star would have spun down³¹ because of angular momentum loss via stellar wind. Eventually in both cases r_p becomes less than r_{CR} and $R_* \approx R_\odot$, causing the

companion to migrate inwards on the timescale $\tau_r \approx 14 \sin i_p$ Gyr, which is much longer than the age of the star for all reasonable values of the inclination angle i_p between the normal to the orbital plane and the line of sight. This is the configuration we observe today. The requirement that the tidal migration timescale (τ_r) be large compared with the life span of a typical solar-type star is a further piece of evidence that supports the interpretation that the companion is a planet with $M_p \approx M_J$ rather than a more massive object. $\square$

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Observation of 'scarred' wavefunctions in a quantum well with chaotic electron dynamics

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QUALITATIVE insight into the properties of a quantum-mechanical system can be gained from the study of the relationship between the system's classical newtonian dynamics, and its quantum dynamics as described by the Schrödinger equation. The Bohr–Sommerfeld quantization scheme—which underlies the historically important Bohr model for hydrogen-like atoms—describes the relationship between the classical and quantum-mechanical regimes, but only for systems with stable, periodic or quasi-periodic orbits¹. Only recently has progress been made in understanding the quantization of systems that exhibit non-periodic, chaotic motion. The spectra of quantized energy levels for such systems are irregular, and show fluctuations associated with unstable periodic orbits of the corresponding classical system^{1–3}. These orbits appear as 'scars'—concentrations of probability amplitude—in the wavefunction of the system⁴. Although wavefunction scarring has been the subject of extensive theoretical investigation^{5–10}, it has not hitherto been observed experimentally in a quantum system. Here we use tunnel-current spectroscopy to map the quantum-mechanical energy levels of an electron confined in a semiconductor quantum well in a high magnetic field^{10–13}. We find clear experimental evidence for wavefunction scarring, in full agreement with theoretical predictions¹⁰.

The semiconductor heterostructure employed in our experiments is the resonant-tunnelling diode (RTD) shown in Fig. 1a. Under an applied bias voltage V , electrons tunnel from a two-dimensional accumulation layer in the left-hand (emitter) contact into the quantum well (QW) formed between the two (AlGa)As barrier layers (Fig. 1b). The magnetic field $\mathbf{B}$ is tilted at an angle θ to the direction normal to the interfaces. To study the classical motion of electrons in the QW, we have calculated electron trajectories for a range of initial velocities at the left-hand barrier consistent with the electron injection energy $\varepsilon_{2\text{DEG}}$. Figure 2 shows Poincaré sections¹ generated by plotting the lateral velocity components (v_x , v_z) each time the electron bounces off the left-hand barrier. At $\theta = 0^\circ$ and $B = 17$ T (Fig. 2a), the electrons execute stable orbits with cyclotron motion about the magnetic field direction, and so (v_x , v_z) lie on concentric circles. When $\theta = 40^\circ$ and $B = 17$ T (Fig. 2b), islands of stable motion coexist with a sea of chaos within which the classical orbits are highly unstable. The size of the chaotic sea increases with increasing B until it fills most of the phase space at sufficiently large fields (Fig. 2c).

The quantized energy-level spectrum corresponding to this regime of predominantly strong classical chaos is shown in Fig. 3a. Close to the energy of the injected electrons (~ 280 meV), most individual energy levels produce well-resolved peaks in the density of levels plot $D(\varepsilon)$. Periodic fluctuations in the density of levels can also be identified by locating the minima (arrowed) between broad peaks in $D(\varepsilon)$. These fluctuations are associated with unstable, but periodic, orbits in the QW via the Gutzwiller trace formula¹.

As V is varied, resonant tunnelling into the energy levels ε_n of the QW might be expected to produce an irregular sequence

of peaks in the current–voltage characteristic $I(V)$. Previous measurements on RTDs, with 60-nm and 120-nm wide QWs in magnetic fields up to 11 T, revealed quasiperiodic series of resonant peaks which were attributed to Gutzwiller fluctuations in the density of levels associated with distinct unstable closed orbits in the QW^{6,7}. In these studies, the QWs were too wide for individual energy levels to be resolved. But our recent theoretical work⁵ indicated that the periodic tunnelling peaks observed for these structures were due to regularly spaced subsets of individual levels, whose eigenfunctions were scarred by particular closed orbits. In the present experiments, the QW is sufficiently narrow (22 nm) that individual levels can be resolved, although a much higher magnetic field is needed to ensure classically chaotic behaviour at $\theta = 40^\circ$.

The current due to resonant tunnelling into the n th state of the QW is determined by the transition rate W_n from the occupied emitter states into the almost empty QW state⁵. In the Bardeen transfer-hamiltonian description^{14–16} of resonant tunnelling,

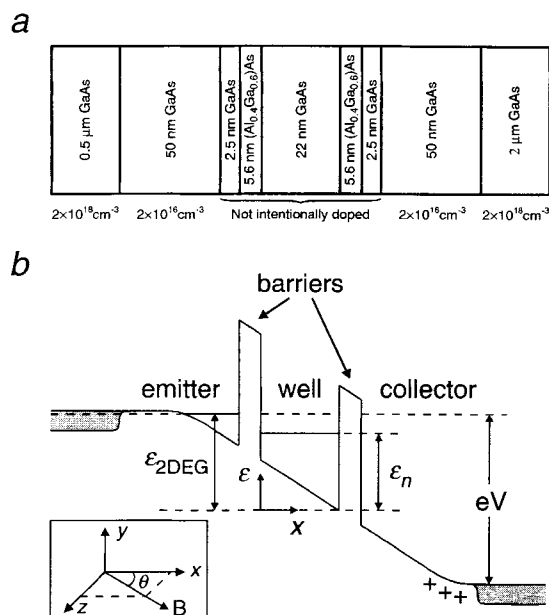


FIG. 1 a, Composition of the resonant-tunnelling diode showing layer thicknesses and concentrations of Si dopant. The semiconductor layers are grown by molecular beam epitaxy and processed into circular mesas with ohmic contacts to the top and bottom doped layers. b, Schematic variation of the potential energy of an electron at the conduction band edge with position x normal to the layer interfaces. The two (Al_{0.4}Ga_{0.6})As layers act as 330-meV-high potential barriers to the motion of electrons through the device. Under an applied bias voltage V , electrons accumulate in the undoped GaAs spacer layer adjacent to the left-hand barrier. The accumulated electrons form a two-dimensional electron gas, in which they are free to move in the plane of the barrier (y – z), but confined within a narrow channel of width ~ 15 nm in the x -direction. This spatial confinement quantizes the energy associated with motion along the x -direction. For the range of V considered here, only the lowest energy level ε_0 is populated. In our calculations, ε_0 is found using a simple model for the potential variation through the device⁶. When a magnetic field $\mathbf{B}$ is applied at an angle θ to the x -direction (see insert) the energy of electrons in the two-dimensional electron gas associated with motion in the y – z plane is quantized into Landau levels. Only the lowest Landau level is populated, and the total energy of the emitter bound state is $\varepsilon_{2\text{DEG}} = \varepsilon_0 + \hbar B \cos \theta / 2m^*$, where m^* is the electron effective mass in GaAs. Electrons in the QW are spatially confined by the two (Al_{0.4}Ga_{0.6})As barriers and by the magnetic field. This confinement quantizes the total energy of an electron in the QW into discrete energy levels ε_n ($n = 1, 2, 3, \dots$). When V is increased, the emitter bound state scans the quantized energy level spectrum of the QW. Resonant tunnelling from the emitter into n th bound state of the QW occurs at the bias voltage for which $\varepsilon_{2\text{DEG}} = \varepsilon_n$.

$W_n \propto M_n^2$, where the matrix element M_n depends on the overlap between the wavefunctions of the emitter and QW states in the tunnel barrier. We have calculated the transition matrix elements using a simple, separable wavefunction for the emitter bound state¹⁵. The variation of M_n^2 with energy ε_n is shown in Fig. 3b. The matrix elements for transitions into a subset of almost equally spaced energy levels (coloured red in Fig. 3a) are much larger than for the remaining states. The reason for this can be seen by examining the probability distributions of the corresponding wavefunctions, two of which are shown in Fig. 3c. These wavefunctions reveal remarkably clear scars of three distinct closed orbits (overlaid) in which the electron makes either two (green

and red curves) or three (blue curve) successive collisions on the right-hand barrier per period. The energies of these scarred states can be accurately located using a Bohr–Sommerfeld quantization of the classical action $S(\varepsilon) = (v + \phi)\hbar$ along the scarring orbits^{4,11} (the different orbits have almost the same classical action for given ε), where $\phi \approx 1$, and the quantum number v gives the number of antinodes in the scar pattern along the classical path⁵. This is similar to the observed scarring of electromagnetic cavity modes by periodic ray trajectories¹⁹. The strong spatial overlap between the emitter state and the subset of scarred states is a direct consequence of the scarring, which localizes the probability density of the QW state in the vicinity of the classical orbit. For

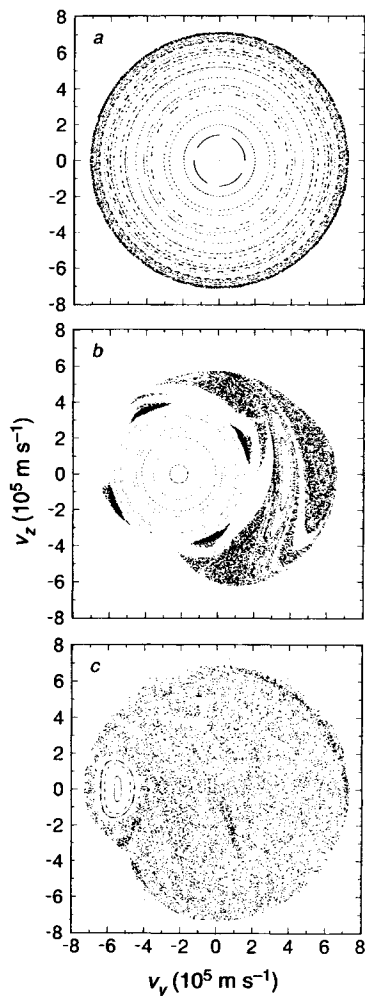


FIG. 2 Poincaré sections showing lateral velocity components (v_x , v_z) for 11,250 collisions on the left-hand barrier (see Fig. 1a) with a, $\theta = 0^\circ$, $B = 17$ T; b, $\theta = 40^\circ$, $B = 17$ T; c, $\theta = 40^\circ$, $B = 37$ T. $V = 668$ mV. Each Poincaré section is generated from 225 starting velocities at the left-hand barrier consistent with the energy $\varepsilon_{2\text{DEG}} \approx 280$ meV of electrons entering the QW from the emitter accumulation layer. Electron orbits within the small island of stability in c collide with the two confining barriers in turn. However, these orbits are inaccessible to the tunnelling electrons and do not affect the electrical properties of the RTD. Non-parabolicity of the GaAs conduction band is included in the classical and quantum calculations by using an energy-dependent effective mass $m^* = 0.067m_0(1 + \alpha K(V))$ where $K(V)$ is the kinetic energy of electrons at the centre of the QW, $\alpha = 2 \text{ eV}^{-1}$, and m_0 is the free electron mass. This value of α was obtained by fitting the positions of resonant peaks in the $I(V)$ characteristics of GaAs/(Al_{0.4}Ga_{0.6})As RTDs containing 22-nm, 60-nm and 120-nm wide QWs with $\theta = 0^\circ$, $\theta = 90^\circ$ (refs 6, 7, 16) and gives m^* values consistent with multi-band perturbation calculations¹⁷.

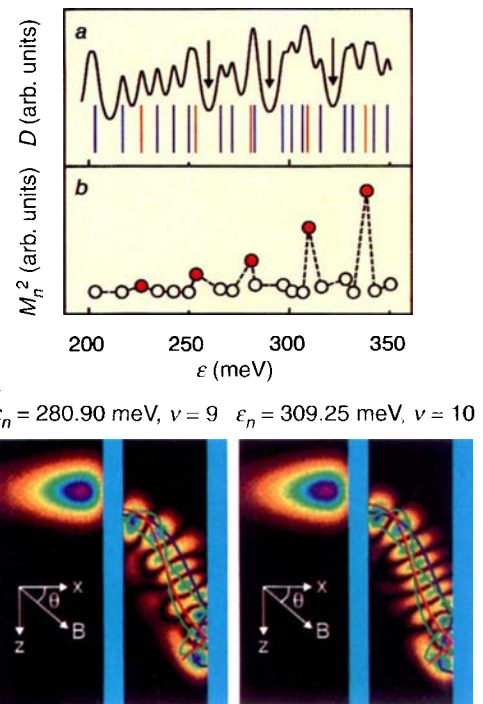


FIG. 3 a, Vertical lines: quantized energy levels ε_n calculated for the QW with $V = 668$ mV, $B = 37$ T, $\theta = 40^\circ$. Details of the calculations are given in ref. 5. Solid curve: density of levels $D(\varepsilon)$ obtained by broadening each energy level to a width of 6 meV consistent with the finite lifetime of ~ 0.1 ps imposed by the emission of longitudinal optic phonons¹⁸. Arrows show successive minima in $D(\varepsilon)$ associated with Gutzwiller fluctuations produced by unstable periodic orbits in the QW in which the electron makes two or three successive collisions on the right-hand barrier for each collision on the left-hand barrier. These orbits also produce strong scars in the wavefunctions corresponding to the subset of energy levels coloured red. Orbits and scar patterns are shown in c. b, Open circles: squared matrix elements M_n^2 for tunnelling transitions into quantized energy levels shown in a. Red circles indicate matrix elements for transitions into a subset of individual scarred states, two of which are shown in c. c, Probability density plots probability increasing linearly from black = 0 through brown, red, orange, yellow, green, blue, dark blue to purple of scarred QW eigenfunctions corresponding to energy levels and quantum numbers v shown. Similar scar patterns are found in the wavefunctions corresponding to all the energy levels coloured red in a. The probability distribution in the emitter accumulation layer is also shown. Blue rectangles indicate positions of barriers. Inset: orientation of magnetic field B in x - z plane. In our chosen gauge given by the vector potential $\mathbf{A} = (0, xB\sin\theta - zB\cos\theta, 0)$, the probability density depends only on the x - and z -coordinates⁵ (axes inset). The trajectories of three distinct unstable periodic orbits which contribute to the scar patterns are shown projected on the x - z plane and distinguished by colour (see text). The energy spacing $\Delta\varepsilon_p \approx 28.4$ meV of the scarred states and also of the Gutzwiller fluctuations in a, is very close to the expected semiclassical value of $\hbar/T_p \approx 28.9$ meV, obtained from the period $T_p \approx 0.143$ ps of the scarring orbits.

unscarred states, the probability is more diffused and the overlap is much weaker.

Figure 4a shows the experimental dI/dV versus V characteristic for $\theta = 40^\circ$ and $B = 37$ T. A regular series of conductance peaks is observed with average voltage spacing $\Delta V \approx 87$ mV. This is much greater than that expected from the mean level spacing in the QW which suggests that the tunnel current is dominated by a subset of levels. We have therefore calculated the tunnelling characteristic from the transition rates studied above. Figure 4b shows the theoretical $I(V)$ curve (black) together with the current contributions due to transitions into individual states in the QW (blue and red). The dominant current contributions (red curves) clearly originate from transitions into scarred states (shown inset) and generate strong resonant peaks in $I(V)$ (arrowed). The predicted peak spacing $\Delta V \approx 94$ mV is in good agreement with the experimental results. This provides clear evidence that the observed resonant peaks originate from transitions into the subset of scarred states shown in Figs 3 and 4b. We stress that the large voltage spacing and periodic distribution of the observed resonant peaks exclude the possibility that they originate from transitions into adjacent energy levels in the QW. A quasi-selection rule based on the periodic scarring of individual wavefunctions is essential to account for the experimental data.

In these experiments, energy- and momentum-conserving tunnelling transitions into a subset of scarred states in the QW have been shown to generate dominant peaks in $I(V)$, which provide direct experimental evidence for the periodic scarring of individual wavefunctions in a nonintegrable quantum system. To our knowledge, this is the only quantum system in which a periodic effect originating from individual scarred eigenstates is observed

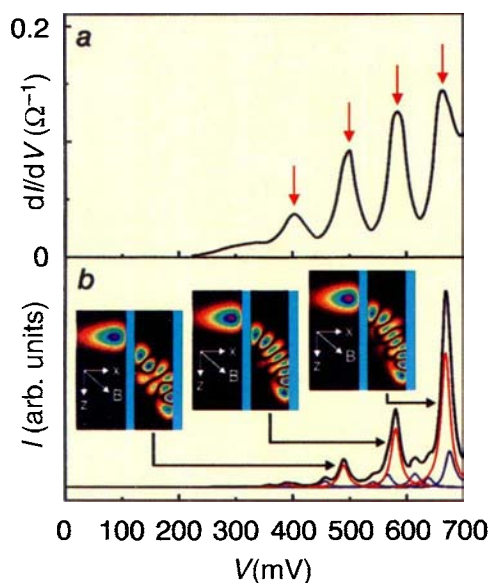


FIG. 4 *a*, Experimental dI/dV versus V plot measured for a 100- μ m-diameter mesa diode at $B = 37$ T and $\theta = 40^\circ$ using a 10-ms pulse magnet at the University of Tokyo. A derivative plot is used to emphasise the resonant peaks (arrowed) which originate from dominant transitions into the scarred states shown in *b*. Transitions into other QW states produce no visible resonant features. No bistability is observed in the tunnelling characteristic, indicating that the occupancies of the QW states are close to zero²⁰. *b*, Calculated $I(V)$ characteristic (black curve) and current contributions (red and blue curves) due to transitions into individual eigenstates of the QW when $\theta = 40^\circ$, $B = 37$ T. Strong periodic resonant peaks in $I(V)$ (arrowed) originate from the dominant current contributions (red curves) produced by tunnelling transitions into individual scarred states with probability distributions in the x - z plane shown inset. These scarred states are calculated at the peak of the current contributions and belong to the same sequence shown in Fig. 3c. The remaining current contributions (blue curves) generate only weak features in $I(V)$.

in experiments. The effect is clearly distinguishable from that due to Gutzwiller fluctuations in the density of energy levels. $\square$

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Photocurrent generation in metal bisphosphonate multilayer thin films

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ATTEMPTS to mimic the highly efficient process of photosynthesis^{1–3} are of considerable interest, the goal being to design artificial systems for the efficient conversion of solar energy into chemical or electrical energy^{4–14}. In both natural and artificial systems, the underlying process is photoinduced charge separation, typically involving a redox reaction between a photo-excited donor molecule and an acceptor molecule. Through careful choice of the molecular arrangement, and the redox potentials of the donors, intermediate charge carriers and acceptors, it is possible to minimize the reverse electron transfer process and thereby obtain stable photoinduced charge separation. In this context, photochemical electron acceptors based on methylviologen and its derivatives have been widely studied^{5,6,8–12,14–19}. Here we describe the synthesis and characterization of metal bisphosphonate multilayer thin films composed of viologen-based acceptor layers and donor layers of *p*-phenylenediamine. Our method of film growth provides control over both the structure and composition of the multilayer films, leading to efficient photoinduced charge separation and directional electron transport. These films generate photocurrents when irradiated with ultraviolet and visible light.

Metal bisphosphonate thin films can be grown either in a layer-by-layer fashion as uniform thin films of constant composition or as a mixed multilayer film with different functions in adjacent

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layers or sets of layers^{15,16,20-24}. We have prepared the zirconium bisphosphonate multilayer films shown in Fig. 1. These films have been characterized by ultraviolet-visible spectroscopy, ellipsometry, cyclic voltammetry, atomic force microscopy and microprobe analysis²⁰. Electronic spectra of the growing films on transparent substrates show a linear increase in absorption as a function of the number of deposition cycles, indicating that the same amount of a given material is deposited with each deposition cycle. Atomic force microscopy studies of thin films of zirconium(1,4-bis(4-phosphonobutylamino)benzene), PAPD, indicate that these films grow uniformly and cover the entire substrate (300-Å-thick films typically show r.m.s. roughnesses of 10 Å or less)²⁰. Although films of N,N'-bis(2-phosphonoethyl)-4,4'-bipyridine, PV, and N,N'-bis(2-phosphonoethyl)-4,4'-bis(4-vinyl pyridine) biphenyl, PVBP, also cover the entire substrate, they grow as a bumpy rather than a flat film. The r.m.s. roughness values for PV and PVBP are 100–200 Å. Given the high roughnesses of these films (of PV in particular), it is clear that they do not grow in a self-limiting, layer-by-layer manner, but rather as closely packed microcrystals seeded on the substrate²⁰.

Irradiation of Au·PAPD/PV electrodes (see Fig. 1 for nomenclature) immersed in an aqueous 0.1 M NaClO₄ solution under anaerobic conditions does not lead to a measurable photocurrent, because there is no oxidizing agent in the cell able to accept electrons from the reduced PV. Figure 2a shows the photocurrent obtained from the Au·PAPD/PV electrode in the presence of a suitable acceptor (Eu³⁺) in solution. When the electrode is exposed to light, a cathodic current is immediately observed, indicating that electrons are photochemically promoted from the PAPD to PV, and then passed to Eu³⁺ in solution. If dissolved oxygen is used as the oxidant rather than Eu³⁺, the current density is significantly lower and the system is less well behaved, that is, the photocurrent gradually increases over time, and ultimately levels off after ~35 minutes. The photoelectrode can then be repeatedly cycled, giving the same current density. The increase in current over time for a cell which uses dissolved oxygen as its oxidant could be due to a structural rearrangement, seen previously for PV films²⁴, which is not observed for a cell which uses Eu³⁺, due to the much higher concentration of Eu³⁺ more effectively trapping the photogenerated reduced species.

Measurement of photocurrents were made in two modes: the potential of the photoelectrode (working electrode) was set to either 0 V versus a Pt counter-electrode (no bias voltage applied) or 0 V versus the standard calomel electrode (SCE), which corresponds to ~-0.4 V bias relative to Pt. The cathodic photocurrents observed were higher with a voltage bias applied (0 V versus SCE) than for no bias voltage (0 V versus Pt) by roughly a factor of two.

The maximum photocurrent observed depends on the film thickness. The largest photocurrents are observed for the films with 1–3 layers each of PAPD and PV. Although making the films thicker increases their optical densities, it also increases the distance that the charges need to travel to leave the film. Charge transfer within a given metal phosphonate layer is easy, and occurs by a self exchange process, facilitated by the close contact of adjacent organic groups within the layer²⁴. The rate-determining process here appears to involve charge transfer between adjacent layers. We are at present investigating thin films prepared with redox-active metal phosphonates, in the hope of increasing the rates of electron transfer between layers.

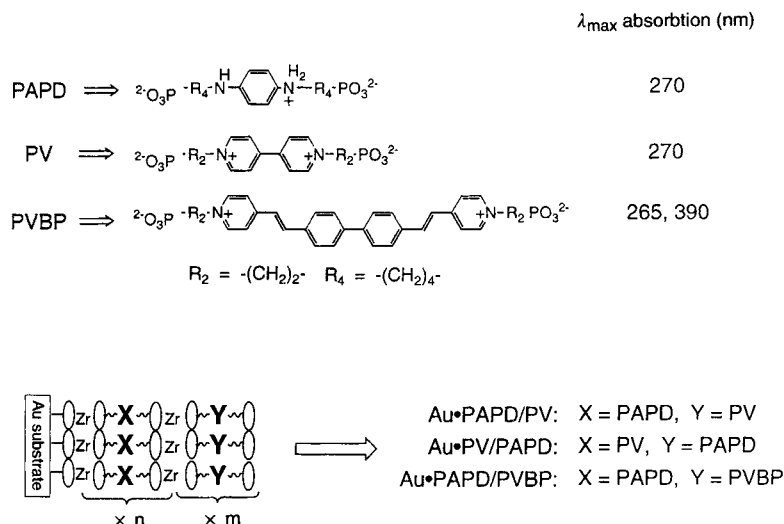
Films prepared with only PAPD or PV produced photocurrents by themselves, as expected for a Schottky diode²⁵. The photocurrents obtained using the Au·PAPD/PV films were roughly an order of magnitude greater than either film alone (of comparable thickness). As a check on the importance of the lamellar heterostructure to the operating properties of the device, we prepared several films (2–6 layers) in which the bisphosphonate growth solution was a mixture of PAPD and PV, leading to molecularly mixed PAPD–PV multilayers. If these films are irradiated photocurrents are not observed (0 V versus Pt), which shows clearly the importance of the segregated lamellar structure. Related metal phosphonates also show efficient photoinduced electron transfer at the donor/acceptor interface¹⁶.

The direction of electron flow can be reversed by switching the order of layers on the electrode. A gold electrode was prepared on which four layers of PV were first deposited, followed by four layers of PAPD. An anodic photocurrent was produced when the film was irradiated, but this current rapidly decayed, owing to the lack of a suitable electron donor in solution. When a donor (EDTA) was added to the solution, a constant anodic photocurrent was observed on irradiation of the electrode (Fig. 2). The anodic photocurrent indicates that electrons are flowing from the film into the gold electrode, as expected for this structure.

These photolyses were carried out with a 220 W Hg/Xe arc lamp (14 mW cm⁻² for wavelengths of 250–300 nm). The maximum current density obtained for the Au·PAPD/PV system was 3.1 μA cm⁻², which corresponds to a quantum efficiency of 0.13% for incident light (250–300 nm) and 4.3% for absorbed light (two layers each of PAPD and PV). The photovoltage at the open circuit is 112 mV. Other workers have built similar lamellar donor/acceptor networks by Langmuir–Blodgett techniques, and have reported comparable quantum efficiencies^{5,22,23}, but the photoelectrodes reported here are significantly more thermally stable. Silicon photoelectrodes display quantum efficiencies (incident light) in the range 2–4% in aqueous systems²⁵.

By replacing PV with other materials, we hope to shift the active

FIG. 1 Schematic representation of the multilayer films studied. The gold substrates were treated initially with 4-thiobutyl-phosphonic acid and placed in a 60 mM solution of ZrOCl₂ for 2 h. The zirconated substrate was then placed in a 5 mM solution of 1,4-bis(4-phosphonobutylamino)-benzene (PAPD) for 3 h at room temperature. Repeated treatments with zirconium and acid solutions led to efficient film growth. Viologen bisphosphonic acid (PV), and the diphenyl (PAV) and the divinylbiphenyl analogues (PVBP) were deposited in a similar fashion to PAPD, but the temperature was increased to 60 °C. ($\lambda_{\max}$ is the wavelength of maximum absorbance.)



wavelengths into the visible portion of the spectrum. PVBP absorbs light at longer wavelengths than PAPD or PV (the wavelength of maximum absorbance, λ_{max} , for PVBP is 400 nm)²⁰. Figure 2b shows the photocurrent produced by irradiation of the Au-PAPD/PVBP electrode, with the lamp filtered such that only wavelengths > 330 nm reached the cell. A large cathodic photocurrent ($\sim 5 \text{ mA cm}^{-2}$) was produced immediately on exposure to the visible light. This current slowly decayed until an equilibrium current density of $1.2\text{--}1.7 \text{ }\mu\text{A cm}^{-2}$ was reached. Once this current density was attained, the system could be repeatedly cycled, with the same current density. The quantum efficiency of the equilibrated photoelectrode (three-electrode configuration) is 0.2%, based on the flux of the incident light (330–800 nm). The photocurrent and the quantum efficiency of photocurrent measured in a two-electrode configuration were $0.4 \text{ }\mu\text{A cm}^{-2}$ and 0.05%, respectively. A photovoltage of 250 mV at the open circuit was measured for this system.

Several physical aspects of these photoelectrodes could be improved, with a view to obtaining significant improvements in quantum yields. Although PAPD acts as a donor in these photoelectrodes, use of a better donor could lead to an increase in photovoltage and quantum efficiency. We are at present examining a range of different donors and sensitizers in the multilayer structures, to try to both increase efficiencies and to shift the active wavelengths into the visible portion of the spectrum. Unfavourable film rearrangement, as well as poor electrical conductivities or junctions, which result in low charge carrier mobility and short carrier lifetime, limit the solar energy conversion efficiencies of our devices. To improve these properties of our materials, we are

working to improve the quality of the metal bisphosphonate films as well as to incorporate redox-active metals to facilitate electron transport through the materials. Solid-state devices are being constructed in an effort to improve their interfacial properties, electron transport efficiency and stability. □

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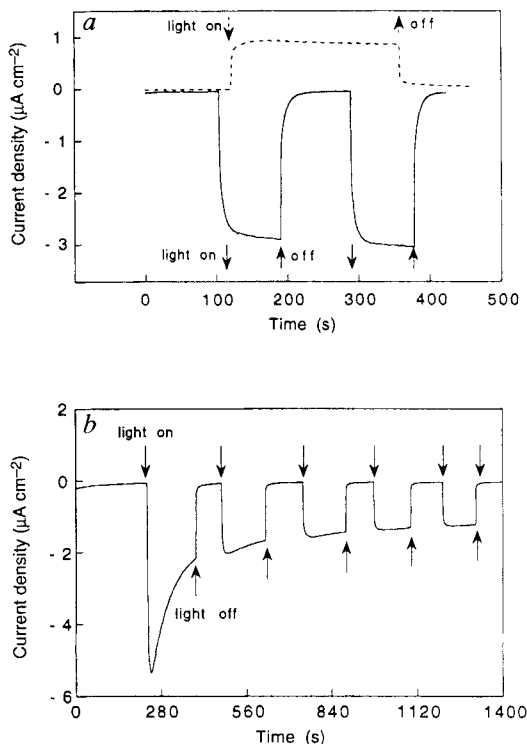


FIG. 2 Photoeffects in Zr bisphosphonate films. a, Cathode photocurrents produced by an Au-PAPD/PV electrode with an electron acceptor ($2 \times 10^{-2} \text{ M Eu}^{3+}$) in solution (solid line) and by an Au-PV/PAPD electrode with an electron donor ($10^{-2} \text{ M Na}_2\text{EDTA}$) in solution (dashed line). The potential of the working electrode was set at 0 V versus SCE in both cases. b, Photocurrents at an Au-PAPD/PVBP electrode with $2 \times 10^{-2} \text{ M Eu}^{3+}$ in solution (illumination through an ultraviolet filter that passed only wavelengths > 330 nm). The potential was set to 0 V versus SCE. All photocurrents were registered using a PAR potentiostat/galvanostat Model 683. The photoelectrochemical cell was kept at 15°C by immersion in a constant-temperature water bath.

Large groundwater inputs to coastal waters revealed by ^{226}Ra enrichments

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THE flow of ground water directly into the coastal ocean has been studied previously by *in situ* measurements, seep meters and diffusion gradient models¹. Although these techniques provide ample evidence that such flows occur, they do not provide a means of quantifying the groundwater flux on a regional scale. Here I report large enrichments of ^{226}Ra in coastal waters of the South Atlantic Bight, and demonstrate that groundwater discharge is the main source of the ^{226}Ra surplus. Using ^{226}Ra data for brackish ground waters with estimates of residence times of nearshore waters, I conclude that the groundwater flux to these coastal waters must be about 40% of the river-water flux during the study period. Besides Ra, other metals, nutrients and organic compounds are expected to be enriched in brackish ground waters, so these findings require an upward revision of terrestrial fluxes of dissolved materials to these coastal waters, and perhaps a re-evaluation of such fluxes to the global ocean. These fluxes may be sensitive to hydrological factors, groundwater usage, dredging and sea-level change.

Activities of ^{226}Ra measured during July 1994 in the South Atlantic Bight (SAB), a region off the southeastern coast of the United States, are shown in Fig. 1. Based on repeated measurements of samples, standards and Environmental Protection Agency cross-check unknowns, I estimate the accuracy for ^{226}Ra to be $\pm 7\%$ at the 95% confidence limit. Internal precision is $\pm 5\%$. Note that the samples within 20 km of shore have activities of 15–28 disintegrations per minute (d.p.m.) per 100 l.

Potential sources of ^{226}Ra to coastal waters include (1) ocean water, (2) river water, (3) desorption from riverine sediments, (4) erosion of terrestrial sediments and (5) ground water. The oceanic source exhibits low variability. Waters seaward of the SAB average 8.0 ± 0.5 d.p.m. ^{226}Ra per 100 l (refs. 2, 3). This is within the range of values measured for salinities $>36.1\%$. Rivers entering the SAB contain lower dissolved ^{226}Ra , of the order of 2–4 d.p.m. per 100 l (refs. 4, 5). Similar activities of dissolved ^{226}Ra in the nearby Savannah and Pee Dee rivers were measured during this cruise.

The accepted reason for higher ^{226}Ra activities in coastal waters relative to rivers and the ocean is the desorption of ^{226}Ra from riverine sediment within the estuary. Li *et al.*² first demonstrated ^{226}Ra enrichments in the Hudson estuary and explained the excess by the loss of ^{226}Ra from the riverine particles. However, desorption cannot explain the enrichments in the coastal waters of the SAB. If the rivers contain 50 mg l^{-1} suspended solids that desorb 2 d.p.m. $^{226}\text{Ra g}^{-1}$ on entering salt water (maximum estimates^{4,5}), only 10 d.p.m. per 100 l could be desorbed. If the river already contained 4 d.p.m. per 100 l, coastal waters should contain 8–14 d.p.m. per 100 l. All of the samples from the SAB inner shelf exceed 14 d.p.m. per 100 l and numerous samples exceed it by a factor of 2.

To estimate more quantitatively the activity of ^{226}Ra supported by rivers, I have used data from US Geological Survey gauging stations on the major rivers that discharge into the study area to determine the amount of riverine water and sediment entering the system. In June 1994, the average total river discharge was $7.0 \times 10^{10} \text{ l d}^{-1}$. Using a suspended sediment concentration of 50 mg l^{-1} (a high estimate) gives a sediment input of $3.5 \times 10^9 \text{ g d}^{-1}$. To summarize; dissolved ^{226}Ra : $(4 \text{ d.p.m. per } 100 \text{ l}) \times (7.0 \times 10^{10} \text{ l d}^{-1}) = 2.8 \times 10^9 \text{ d.p.m. d}^{-1}$; desorbed ^{226}Ra : $(2 \text{ d.p.m. g}^{-1}) \times (3.5 \times 10^9 \text{ g d}^{-1}) = 7.0 \times 10^9 \text{ d.p.m. d}^{-1}$; total, $1 \times 10^{10} \text{ d.p.m. d}^{-1}$.

This study stretches from the Cape Fear River to the Savannah River, a distance of 320 km. The highest ^{226}Ra activities are found in the waters above the inner shelf which extends about 20 km from the shoreline. A strong pycnocline at 10 m depth effectively isolated the surface waters from the underlying waters. These parameters yield a volume of $6.4 \times 10^{13} \text{ l}$ for the zone of highest ^{226}Ra enrichment. The riverine input can support

$$\frac{1 \times 10^{10} \text{ d.p.m. d}^{-1}}{6.4 \times 10^{13} \text{ l}} = 0.016 \text{ d.p.m. per } 100 \text{ l per day}$$

To determine the ^{226}Ra balance, the residence time of water on the inner shelf must be known. Atkinson *et al.*⁶ used the freshwater balance to estimate an 80-day shelf-flushing time in the Georgia Bight. Similar flushing rates were estimated for Onslow Bay, North Carolina⁷. The flushing rate is the time required to remove fresh river water from the entire 100-km-wide shelf. The value selected for the residence time is not very important in evaluating the riverine source of ^{226}Ra . Using a residence time of 30 days for the inner 20 km of the shelf would allow the riverine input to produce a ^{226}Ra enrichment of 0.5 d.p.m. per 100 l. Compare this to average ^{226}Ra enrichments (measured activity minus ocean activity) in this zone of 11 d.p.m.

TABLE 1 ^{226}Ra activities in lower-coastal-plain ground water

Site, no of wells	Salinity (‰)	^{226}Ra (d.p.m. per 100 l)		
		Range	Mean	Reference
Myrtle beach, 9	0	4–14	7	10
Surfside, 2	0	11–13	12	10
Beaufort, 9	0	4–50	24	10
Hilton Head, 5	0	10–76	40	10
Charleston, 4	7–24	130–530	300	This work
North Inlet, 1	0		66	This work
North Inlet, 4	15–22	710–840	770	This work
North Inlet, 7	25–33	90–150	120	9

per 100 l (Fig. 1). During this study period, rivers supplied at most 5% of the ^{226}Ra enrichment in the SAB. It would be difficult to increase this estimate. Suspended sediment input, ^{226}Ra desorption and inner-shelf residence times are high estimates; furthermore, this calculation does not consider ^{226}Ra enrichments that occur seaward of 20 km.

In the deep sea, ^{226}Ra is supplied by desorption of regenerated activity. Regeneration of ^{226}Ra activity on sediments from which it has desorbed proceeds as:

$$A^{226}\text{Ra} = A^{230}\text{Th}(1 - e^{-\lambda_{226}t})$$

where λ_{226} is the decay constant of ^{226}Ra ($4.33 \times 10^{-4} \text{ yr}^{-1}$) and A is activity of the parent and daughter nuclides. Thus, 500 years are required to regenerate 20% of the desorbed activity; that is, $A^{226}\text{Ra}/A^{230}\text{Th} = 0.2$. Therefore, marine sediments that have desorbed ^{226}Ra contribute little to enrichments of ^{226}Ra in the coastal ocean.

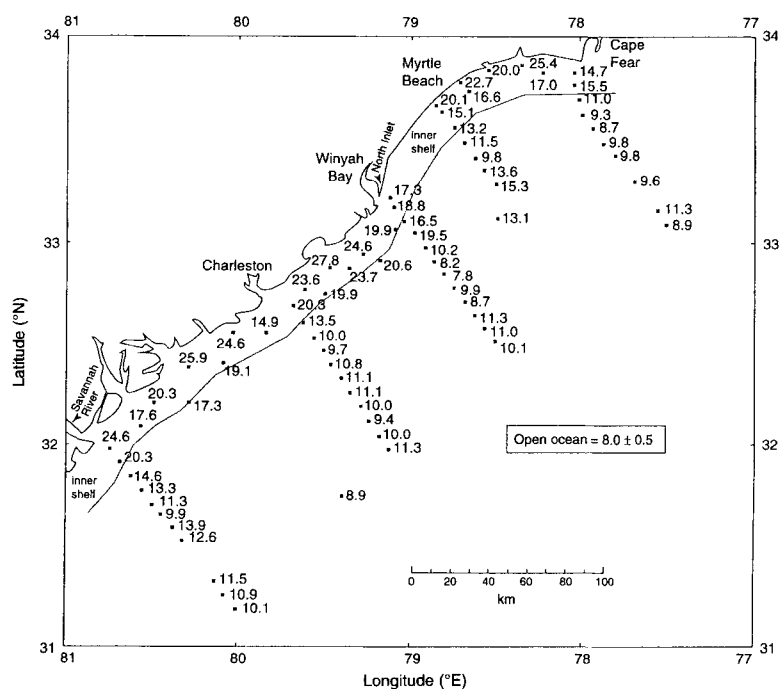


FIG. 1 Activities of ^{226}Ra (d.p.m. per 100 l) measured in surface waters of the South Atlantic Bight (SAB) from 8 to 11 July 1994. Surface samples (100–350 l) were filtered through a $1.0\text{-}\mu\text{m}$ -pore cartridge and a Mn-fibre column to concentrate Ra (ref. 15). Samples were leached with HCl in a Soxhlet extraction apparatus to remove Ra. The Ra was co-precipitated with BaSO_4 and aged to allow equilibrium to be established from ^{226}Ra through ^{214}Bi . γ -rays were measured in a well-shaped germanium detector to determine the activity of ^{226}Ra (ref. 16).

To explain ^{226}Ra enrichments in Chesapeake Bay, Maryland, which exceeded river inputs, Helz *et al.*⁸ suggested that shore erosion would provide additional desorbable ^{226}Ra . This could be a source in the SAB during periods of severe coastal erosion; however, the May–June 1994 period was not stormy. If this mechanism was important during this study, a mass of fine-grained sediment many times greater than the suspended riverine input that had not been exposed to sea water for hundreds of years, must have been put into suspension in the water column in the month preceding the cruise. Although this potential source is difficult to evaluate, it is probably minor.

By process of elimination, the primary source of the ^{226}Ra enrichment must be the discharge of ground water containing dissolved ^{226}Ra . A similar conclusion was reached by Rama and Moore⁹ who estimated the amount of ^{226}Ra exported from the North Inlet salt marsh. In this system which has insignificant fresh surface water input, the export of ^{226}Ra could be supplied by an average groundwater flow of 10 cm d^{-1} over the marsh surface.

King¹⁰ measured ^{226}Ra in a number of drinking-water wells in the lower coastal plain. None of these contained especially high activities, but all were above the activity in river water. Considerably higher ^{226}Ra activities occur in sediment pore waters (salinity 25–33‰) in the North Inlet salt marsh⁹. New samples of brackish (salinity 7–22‰) coastal ground waters reveal substantially higher activities, in the range 100–800 d.p.m. per 100l (Table 1).

I hypothesize that salt water contamination of coastal aquifers leads to desorption of ^{226}Ra and enhanced groundwater activities, similar to the data in Table 1. Note that the brackish samples having 700–800 d.p.m. per 100l are within 50 m of the freshwater well containing only 70 d.p.m. per 100l. Progressive movement of a salt front into an aquifer and discharge of the brackish water into the near shore zone will provide a source of ^{226}Ra to the coastal ocean. This source will be important as long as there is continued advancement of the salt front into the aquifer and a net advection of brackish ground water into the ocean. Factors leading to salinization of aquifers include high utilization of ground water to support a growing coastal population, rise of sea level, and breaching of upper confining beds by dredging. A report of the South Carolina Water Resources Commission¹¹ concluded that all of the uppermost aquifers along the South Carolina coast are contaminated by sea water. From a high-resolution seismic survey, Duncan¹² noted that many of the shallow aquifers are within a few metres of the surface and are vulnerable to breaching by dredging. Although groundwater mining may have reduced the total groundwater discharge to the coastal ocean, the discharge of ^{226}Ra could be substantially greater owing to its desorption as the salt front advances inland. This mechanism is expected to produce inputs of ^{226}Ra wherever the confining beds are fractured or absent, whether by natural causes or by dredging.

We can use the assumptions of the earlier calculation and the data in Table 1 to predict the amount of ground water necessary to produce the ^{226}Ra enrichments. The average ^{226}Ra activity of inner-shelf waters is 19 d.p.m. per 100l (Fig. 1). At most, 1 d.p.m. per 100l is derived from desorption in estuaries and 8 d.p.m. per 100l from the ocean. Therefore, the required excess

^{226}Ra input is:

$$\frac{(10\text{ d.p.m. per } 100\text{ l}) \times (6.4 \times 10^{13}\text{ l})}{30\text{ days}} = 2.1 \times 10^{11}\text{ d.p.m. d}^{-1}$$

If the groundwater ^{226}Ra activity is similar to the brackish ground water in North Inlet, 700 d.p.m. per 100l, a volume of $3 \times 10^{10}\text{ l d}^{-1}$ ($350\text{ m}^3\text{ s}^{-1}$) is required to support the excess ^{226}Ra on the inner shelf. Compare this to the average river flux of $7 \times 10^{10}\text{ l d}^{-1}$ during June 1994 to appreciate that the required groundwater flow is of the order of 40% of the river flow. Also ^{226}Ra enrichments beyond the inner shelf have not been considered.

These summertime data cannot be extrapolated to average annual conditions. Moore¹³ observed that ^{226}Ra activities on the inner shelf near Brunswick, Georgia, were 10–14 d.p.m. per 100l in February and April 1982, but increased to 12–24 d.p.m. per 100l in August 1982; samples collected near Winyah Bay in August 1982 ranged from 17–25 d.p.m. per 100l. The August 1982 data at both sites are in the range of values for July 1994; the winter and spring values are considerably lower. Similar seasonal differences occur in North Inlet¹⁴ where 27–39 (mean 32) d.p.m. ^{226}Ra d.p.m. per 100l were measured in July 1984 compared to 14–32 (mean 22) d.p.m. per 100l for four sampling periods during non-summer months. Thus, summer seems to be a time of increased ^{226}Ra enrichments and presumably of groundwater flow.

Simmons¹ reported submarine groundwater fluxes of $5\text{--}20\text{ l m}^{-2}\text{ d}^{-1}$ off the coast of both North and South Carolina. These fluxes are about 10% of the estimated North Inlet flux⁹. An average flux of $5\text{ l m}^{-2}\text{ d}^{-1}$ over the inner shelf would balance the ^{226}Ra enrichment.

The ^{226}Ra signal provides a sensitive tracer of groundwater input to the coastal ocean because we can characterize other inputs well. Many other elements and ions are expected to have similar geochemistry, and to desorb into brackish ground water. Important constituents include barium, caesium, phosphate, ammonia and cadmium. Other elements may be enriched in brackish ground waters due to variable redox conditions. These include manganese, iron, uranium, chromium and vanadium, as well as species adsorbed on Mn and Fe oxides. Concentrations of these elements in ground water may be orders of magnitude higher than in river water. The groundwater flow may largely bypass the estuary filter and deliver the elements directly to the ocean. If groundwater input is as high as the ^{226}Ra data require, a re-evaluation of terrestrial fluxes to the oceans is required. Furthermore, groundwater inputs will vary with sea level. During sea-level rise, salt-water intrusion into coastal aquifers will release desorbable components. Transitions from glacial to interglacial conditions may be accompanied by fresh inputs of phosphate and barium to the ocean. Such inputs have not been considered for these important palaeoceanographic tracers. The ^{226}Ra signal may also provide a means of identifying an aquifer undergoing salt intrusion. The signal is produced as salty water penetrates into uncontaminated sediments and is lost as the aquifer flushes. Unless the salt front is advancing, no signal is expected unless the aquifer is stagnant. □

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A Triassic Lagerstätte from eastern North America

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THE end of the Triassic period is pivotal in the evolution of modern ecosystems¹. Despite this, the Triassic remains one of the poorest known periods in the evolutionary history of the terrestrial arthropods. Here we report on fossiliferous shales preserving a nearly complete marginal lacustrine community from the Virginia–North Carolina border that sheds considerable light on this critical interval. Three species of insect were previously described from this locality, but the full extent and significance of its diversity have only now been discovered: we report here the oldest definitive records for three orders of insect and numerous families and superfamilies. Furthermore, in addition to new taxa, the flora is shown to contain an unusual diversity of forms, some of which have only been previously reported either from Europe or the Southern Hemisphere. The abundance of complete insects and the preservation of soft-part anatomy on some of the vertebrates elevates the site to one of the most significant Lagerstätte in the world.

A series of markedly cyclical lacustrine sediments of the Carnian Cow Branch Formation are exposed at the Solite Quarry, Cascade, Virginia, USA² (Fig. 1), and these are fossiliferous throughout. The cycles are thought to represent periodic changes in lake depth under the control of known variations in the Earth's orbit (Milankovitch cycles)³. The sediments deposited in the shallowest water contain footprints and abundant plant fragments, together with mud cracks and ripple marks. However, the richest horizons are microlaminated, organic-rich shales deposited in the deepest water, and it is in these sediments that a great diversity of insects has been found. Articulated remains of the tanytropheid reptile, *Tanytrachelos*, complete with impressions of soft tissue, also occur in these horizons, together with the best preserved plant remains.

Although over 30 cycles are exposed in the two main quarries, one cycle has proved to be particularly productive and most of the insects have been recovered from a thin band of microlaminated sediments at the base of this unit. Nevertheless, at least three other cycles have yielded insect remains. The depositional environment has been interpreted as a chemically stratified (meromictic) lake with a well-oxygenated epilimnion but an anoxic hypolimnion⁴. The anoxic bottom waters would have been largely devoid of life, as evidenced by the lack of bioturbation and decay. The matrix is an exceptionally fine-grained black shale, and the insects are preserved as two-dimensional silvery images. Microscopic details are preserved with great fidelity and the resolution of preserved detail is ~1 µm. Even microtrichiae on the body and wings of insects 2 mm long are sometimes preserved.

A complete list of the insect groups represented in the Solite assemblage is given in Table 1b. The most abundant insects are aquatic heteropterans (water bugs). Two families, the Belostomatidae and the Naucoridae, are

represented by numerous nymphal and adult specimens (Fig. 2a). These are the oldest definitive records of nepomorph Heteroptera, which include many of the predatory aquatic Heteroptera.

After the water bugs,uchenorrhyncans (leaf-hoppers) are the most common order of insect in the assemblages. Typically the wing venation in the new specimens (Fig. 2b) most closely resembles the Archescytinidae, which is known from the Permian period of Kansas, Russia and New South Wales. Two isolated wings exhibit a very unusual venation (Fig. 2c), which by elimination are considered most likely to belong to uchenorrhynchans.

A single specimen of Thysanoptera (thrips) (Fig. 2d, e) preserves short wings that end at the anterior margin of the penultimate tergite. Marginal fringes are present on both the forewings and hindwings. *Permothrips longipennis* has been described as the oldest thrips⁵, but this referral is based purely on the body form and wing shape. In *Permothrips*, the wings extend well beyond the apex of the abdomen and they lack the fringed margins—conditions not found in any modern thrips. Consequently, the Solite thrips is the oldest definitive specimen in this order. Furthermore, the next oldest thysanopteran, *Liassothis crassipes*, from the Jurassic period of Russia, is very poorly preserved⁶: the wings are missing or it may even be a nymph. The only other well-preserved Mesozoic thysanopterans occur in Aptian (Lower Cretaceous) amber from Lebanon⁷.

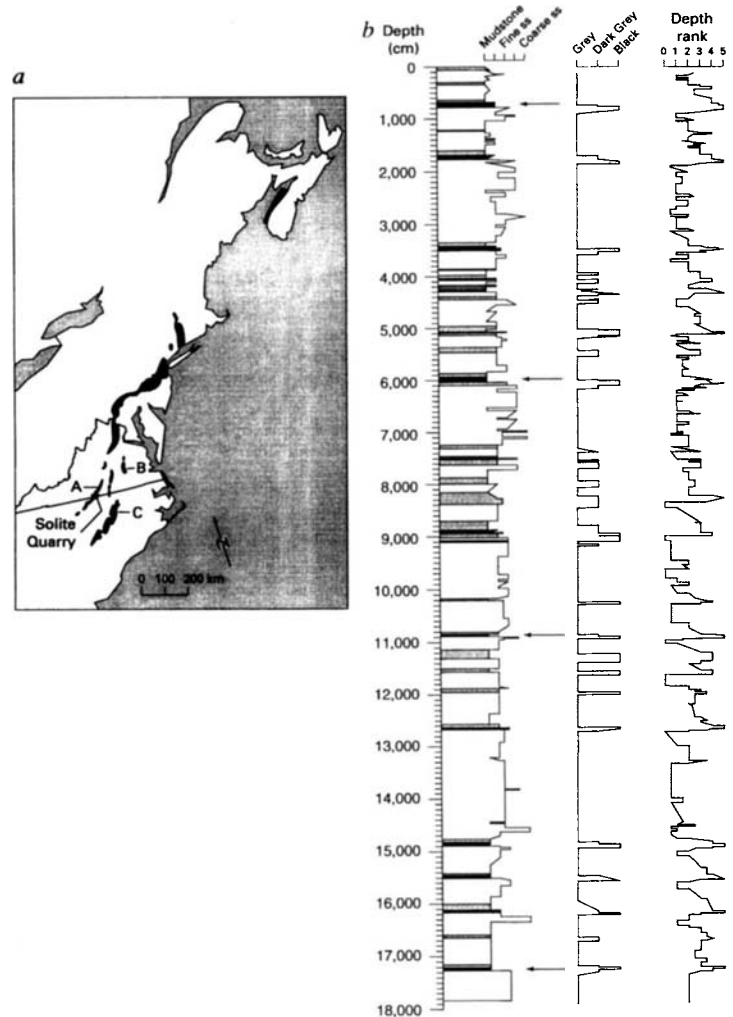


FIG. 1 a, Map showing the Triassic basins of the Newark Supergroup and the Solite Quarry. A, Dan River/Danville basin; B, Richmond basin; C, Deep River basin. b, Geological section through the first of the two main quarries. Arrows indicate cycles known to produce insect remains.

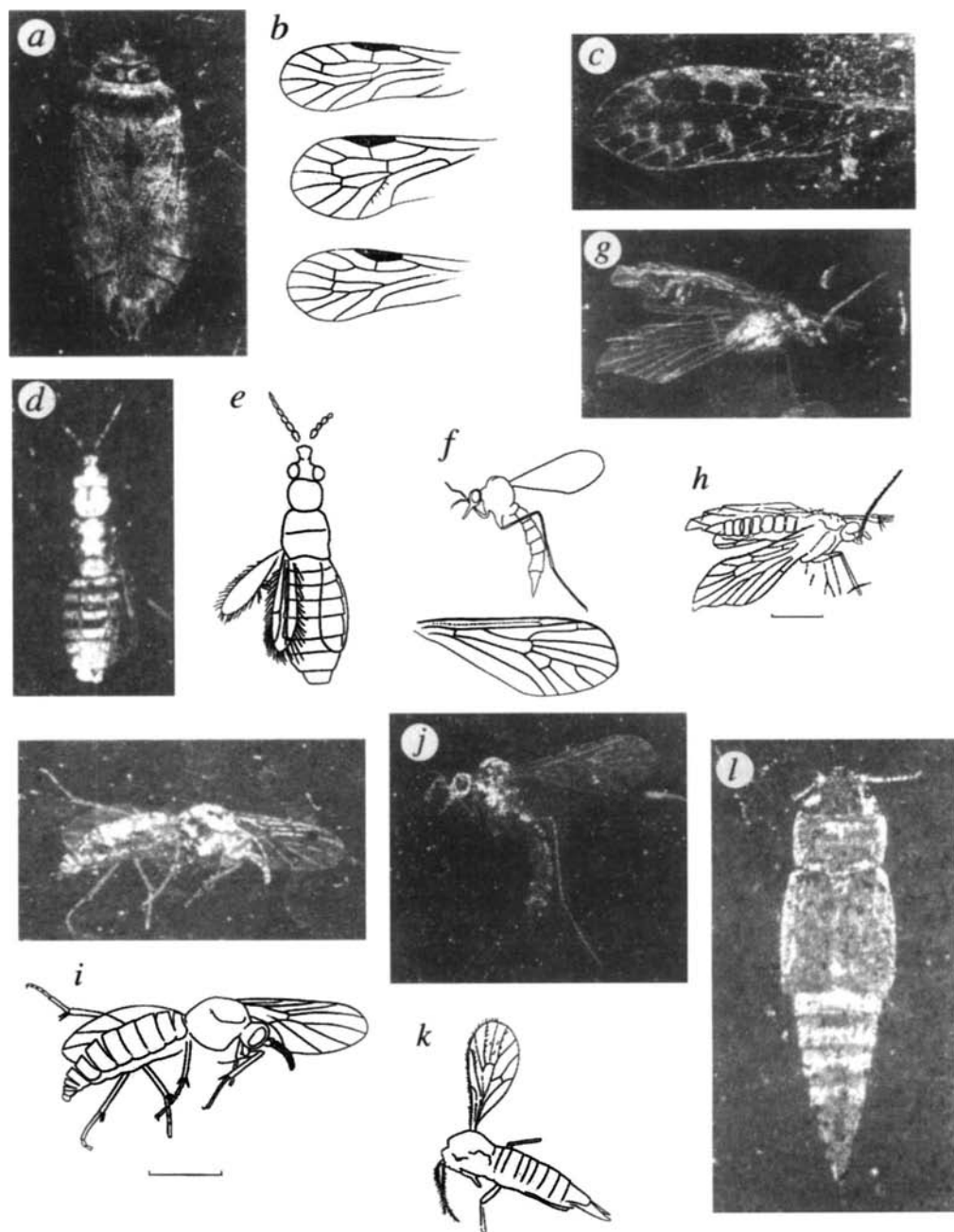
A single complete specimen of a trichopteran (caddis-fly) is known from the Solite assemblage (Fig. 2g, h). Although trichopterans have been described from the Permian and Triassic of Russia⁸, in none is the body as well preserved as the present specimen, and the wing venation of those is considerably more primitive than the new form and later Mesozoic taxa.

The oldest definitive dipteran (fly) is from the Lower/Middle Triassic of France⁹, but the dipterans in the Solite assemblage constitute the next oldest record of this order, and are very diverse, with at least six families represented. Three of these were recognized in the original material collected from the quarry in the 1970s (ref. 10), although their precise referrals need to be revised. For instance, Krzeminski¹⁰ placed *Alinka* in

the Brachycera, but the wing venation is actually characteristic of the Bibionomorpha (March flies), and only nematocerous flies have been identified so far. Tipulids have the oldest fossil record of all the dipteran families, which may reflect their status as the sister group to all other flies. They are represented in the Solite assemblage by excellent specimens of *Architipula youngi* (Fig. 2f, j).

Of the new material, a particularly important find is of two individuals of *Crosaphis*, which was originally described from an isolated wing from the upper Triassic of Australia¹¹. There has been considerable uncertainty concerning the ordinal assignment of *Crosaphis*. First described as an aphidoid hemipteran¹¹, then referred to the coccoids (scale insects) by Heie¹² and the Diptera by Kovalev¹³, these referrals did not receive wide acceptance

FIG. 2 Insects from the Solite assemblage. a, One of the numerous belostomatid specimens (VMNH 727), magnification $\times 4.6$. Like living belostomatids, the Solite belostomatids have hind tarsi with a fringe of setae and coriaceous forewings. The forewings differ from modern forms, however, by the intricate, reticulate wing venation: the most primitive condition seen so far in this group. b, Variation in forewing venation pattern observed in three different specimens of auchenorrhynchan. In all examples the wing venation most closely resembles that of the Archescytinidae. However, typically in archescytinids the forewings and hindwings are similar in size, but in the new specimens the hindwing is much narrower than the forewing. c, Isolated wing (VMNH 728) attributed to an auchenorrhynchan, magnification $\times 6$. The venation is very odd for its single longitudinal vein running parallel to the length of the wing, from which obliquely split off a row of eight veins parallel to each other. The wing is patterned, with a sclerotized costal area and clouds over each crossvein, and is unlike any other previously described. d, e, The single thysanopteran specimen (VMNH 729), magnification $\times 25$, which preserves the antennae, head, thorax, abdomen and two pairs of wings in their entirety. The rudimentary venation on the forewing is not unlike that of some primitive, modern-day thrips. f, *Architipula youngi* (VMNH 732), magnification $\times 10$. g, VMNH 730, an almost complete trichopteran, magnification $\times 15$. h, Camera lucida drawing of the trichopteran showing details of the head, body, some legs and most of the wings. Note in particular the characteristic tibial spurs and microtrichiae; scale bar, 1.0 mm. i, One of the two specimens of *Crosaphis*, VMNH 731; scale bar, 1.0 mm. j, *Architipula youngi* (VMNH 732) showing details of the wing venation pattern. k, A psychodoid fly, VMNH 733, magnification $\times 14$, showing the head, antennae with long setulae, body, some legs and a complete wing. Diagnostic features of the wing essential for accurate placement are clearly preserved and include the wing apex broadly rounded, vein Sc turned abruptly costad, veins M1 and M2 forked, forked cubital vein,



and veins with long setae. l, One of the three staphylinid beetles, VMNH 734, in the Solite assemblage, magnification $\times 30$. Microtrichiae on the head, thorax and elytra are beautifully preserved. The specimens have been assigned to the Staphylinidae on the basis of the distinctively short elytra (exposing six or seven abdominal tergites), and the pronotal and head shapes.

TABLE 1 List of taxa from the Solite Quarry

(a) Plant taxa	(b) Insect taxa
Lycopodiales (lycopods)	Auchenorrhyncha (plant hoppers, leaf hoppers)
<i>Lepidodendron</i> type	Archescytinidae
<i>Lepacyclotes</i> sp.	Wing from undetermined family
Arthrophyta (scouring rushes)	Blattodea (cockroaches)
<i>Neocalamites</i> cf. <i>knowtonii</i>	undetermined family
Pteridophyta (ferns)	Coleoptera (beetles)
<i>Lonchopteris virginienensis</i> (cf. <i>Cynepteris synorensis</i>)	Schizophoridae
<i>Cyathoforma</i> sp.	Staphylinidae
<i>Dictyophyllum</i> sp.	Diptera (flies)
<i>Wingateia</i> sp.	Tipulidae (crane flies)
cf. <i>Achrostichites linnaeaeifolius</i>	Crosaphidae
Ginkgophyta (ginkgos)	Procramptonomyiidae
<i>Sphaenobaeria</i> sp.	Psychodidae (moth flies)
<i>Metreophyllum</i> sp.	Eoptychopteridae
Coniferales (conifers)	Heteroptera (sucking bugs)
cf. <i>Hirmeriella</i> sp.	Belostomatidae
<i>Pagiophyllum simpsoniae</i>	Naucoridae
<i>Pagiophyllum diffusum</i>	Orthoptera
<i>Pagiophyllum</i> sp.	undetermined family
<i>Brachyphyllum</i> sp.	Thysanoptera (thrips)
cf. <i>Composstrobis neotericus</i>	undetermined family
cf. <i>Elatocladus</i>	Trichoptera (caddis-flies)
<i>Podozamites</i> sp.	Undetermined family
Anthophyta	(c) Fish taxa
Bennettitales (cycad-like seed plants)	Actinopterygii
<i>Zamites powelli</i>	Palaeonisciformes
<i>Pterophyllum</i> sp.	<i>Turseodus</i> sp.
<i>Sphenozamites</i> sp.	<i>Synoricthys</i> sp.
Cycadales (cycads)	<i>Cionichthys</i> sp.
cf. <i>Zamiostrobus lissocardus</i>	Semionotidae
Incertae sedis	<i>Semionotus</i>
<i>Pannaulika triassica</i>	Sarcopterygii
<i>Fraxinopsis</i> sp.	Coelacanthini
<i>Pelourdea</i> sp.	<i>Diplurus</i> cf. <i>Newarki</i>
Seeds with dispersal hairs	cf. <i>Pariostegus</i>

because of the fragmentary nature of the material¹⁴. The new specimens, preserved in entirety (Fig. 2i), show *Crosaphis* to be indeed a dipteran, specifically in the family Anisopodidae (woodgnats) and related to the living genus *Mycetobia*. The similarity in the wing venation between the Australian and Solite specimens is striking, especially in light of the great palaeocontinental distances separating the two Triassic deposits.

Another important find is a single specimen (Fig. 2k) in the living superfamily Psychodoidea (moths and sandflies). The specimen either represents a new family very closely related to the Psychodidae, or it is the most primitive known member of the family. The next oldest specimens in this group were recently described from the Jurassic period of Germany¹⁵.

With representatives of four living infraorders of the Diptera found so far in the Solite assemblage (Bibionomorpha, Psychodomorpha, Tipulomorpha and Ptychopteromorpha), this deposit has uniquely revealed an ancestry of the Diptera more ancient than commonly believed. The placement of *Permotipula*, from the Permian of Australia, as the oldest dipteran or closest relative now seems less controversial¹⁶.

Like the Diptera, the Coleoptera from the Solite assemblage are the oldest beetles from the New World (the oldest beetles are from the Permian of Russia¹²). A single large Solite specimen is in the extinct family Schizophoridae, and in fact possesses features clearly allied to taxa from the Triassic of Russia¹⁷. The Schizophoridae belongs to an assemblage of 12 families, the Cupeodoidea, of which all except one are extinct. The cupedoids were the dominant coleopteran in the late Palaeozoic and early Mesozoic eras. More exciting are three specimens unquestionably belonging to the huge living family, the Staphylinidae (rove beetles; about 30,000 species have been described) (Fig. 2l). Before this find the oldest staphylinids were from the Jurassic of Russia, to which the Solite specimens bear a distinct resemblance¹⁸. Modern staphylinids are predominantly inhabitants of

forest leaf litter.

Despite lacking cuticle (typically a diagnostic feature of fossil plants), the plant remains (Fig. 3a–d, f) show a remarkable diversity (Table 1a). Ferns, cycadeoids and conifers predominate, and for a single palaeobotanical site, the Solite quarry is as rich as any in the world. The ferns in general are biostratigraphically and palaeogeographically significant. A single specimen is referable to *Wingateia* (Fig. 3a), a form well documented from the upper Carnian Deep River basin and the Chinle^{19,20}. However, the presence in the Solite assemblages of *Lonchopteris virginienensis* (= *Cynepteris synorensis*) and *Cyathoforma* is much more consistent with the floral assemblages from the slightly older (Julian and Tualian) Richmond basin and the Santa Clara Formation of New Mexico, rather than the Deep River basin and Chinle (where *Cynepteris lassiphora* is the typical form).

Specimens of ginkgophyte foliage show a remarkable similarity to *Metreophyllum*. *Metreophyllum* has been described only from the Permian of South Africa. It is essentially very like the European Jurassic genus *Eretmophyllum*, but significantly larger. The new find is the first record of an *Eretmophyllum*–*Metreophyllum* type of foliage for North America, and it hints at a possible Pangaeian distribution.

Bennettitales, which could represent early members of the anthophyte radiation, are well represented by articulated fronds and leaf fragments (Table 1a). The referral of *Pannaulika*, previously described from the quarry, to the angiosperms²¹ is potentially even more significant. Although there is cause for some scepticism regarding this determination, the looping tertiary venation pattern is certainly angiosperm-like. Although the dipteridaceous fern *Dictyophyllum* also exhibits a somewhat angiosperm-like venation, it is quite unlike that of *Pannaulika* (Fig. 3f).

Two specimens of a rather unusual seed are here referred to the genus *Fraxinopsis* (Fig. 3c, d). The multiple parallel veins on the

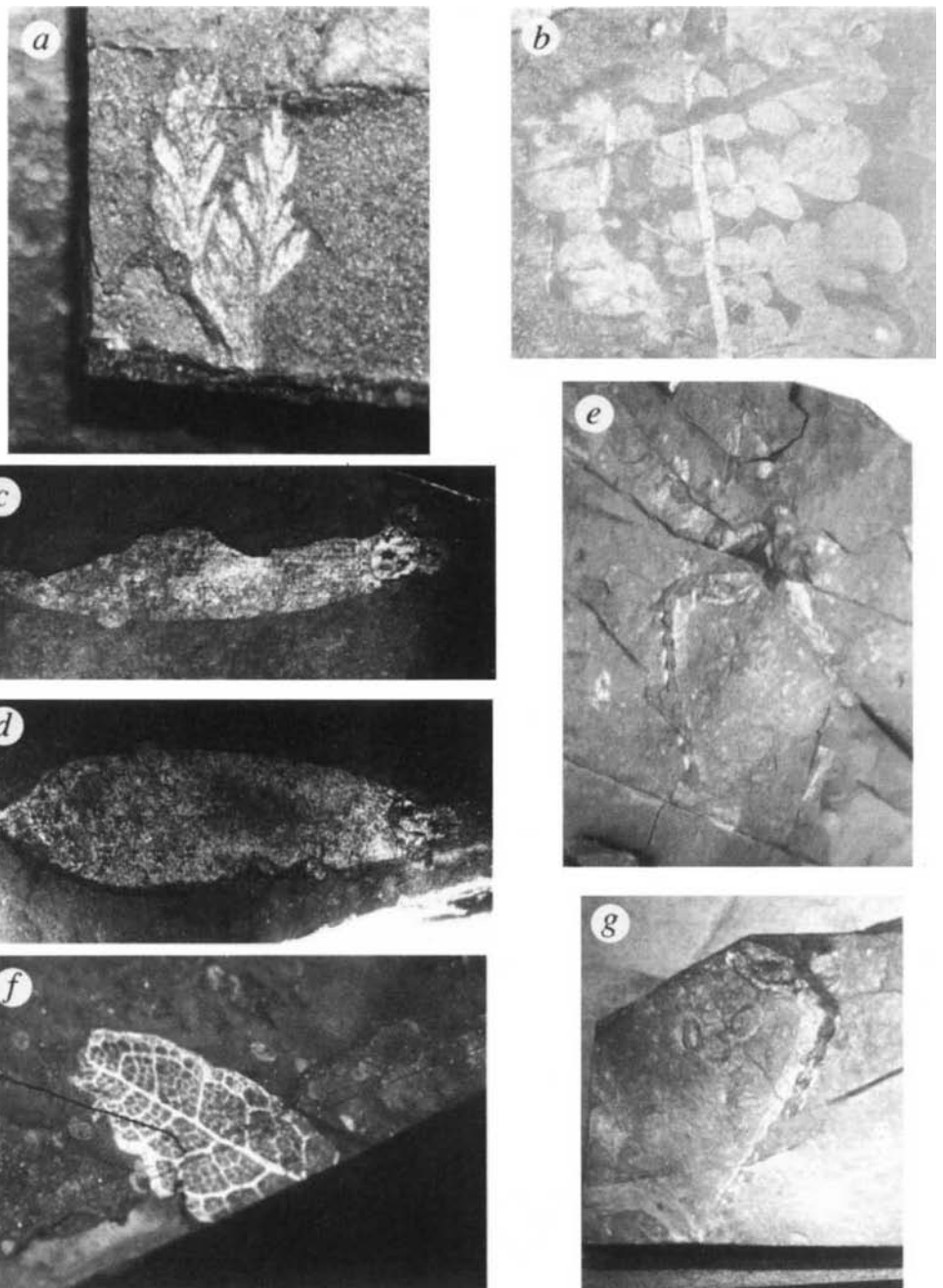


FIG. 3 a, Foliage referred to *Wingatea* (VMNH 735), magnification $\times 6$. b, A previously undescribed fern-like foliage (VMNH 736), magnification $\times 2$. It is represented by many specimens and is notable for the much enlarged apical pinnule. c, d, The two specimens of seed referred to *Fraxinopsis* VMNH 737 (c) and VMNH 738 (d). e, The caudal portion of a *Tanytrachelos* tail showing the vertebrae and myotome muscle blocks (VMNH 739), magnification $\times 1.0$. f, *Pannaulika triassica* (VMNH 201) magnification $\times 2.8$. g, *Tanytrachelos ahynis*, counterpart of VMNH 739, magnification $\times 1.0$.

seed lamina are very characteristic, and this should therefore be regarded as the first occurrence of this taxon from Laurasia.

The aquatic prolacertiform tetrapod *Tanytrachelos* has already been described from many articulated specimens from the Solite Quarry. But many more specimens have been recovered in the latest excavations, and these include some spectacular individuals complete with ghosts of the myotome muscle blocks on the tail (Fig. 3e, g) and ligaments in the webbed hind foot. Fragmentary remains of two as-yet undescribed tetrapods have also been found. In addition to the fishes previously described (Table 1c), we have also recovered an isolated shark tooth which, at 8 mm, is much larger than most freshwater shark teeth.

Besides the spectacular preservation and the number of unique taxa, the new finds have a bearing on debates about the modes of terrestrial evolution across the Triassic–Jurassic boundary. In an analysis of insect diversity over time, Labandeira and Sepkoski²² indicated that the Triassic was a period of unexpectedly low diversity, and they suggested that this reflected either increased extinctions or slowed diversification. With the finding of seven

new, earliest records of living insect families/orders from the Solite assemblage, based on just over 200 specimens, this suggests that a 'Triassic minimum' of insect diversity is a sampling artefact due to the paucity of Triassic deposits.

The homogeneity of Early Jurassic terrestrial vertebrate faunas has been widely recognized^{23–25}, but in the Late Triassic, despite the presence of a single supercontinent, Pangaea, some localization (partitioning) of vertebrate faunas has been reported, with particularly marked distinctions between Northern (Laurasian) and Southern (Gondwanan) Hemispheres. This same separation has also been recognized for plants²⁶. However, some doubt was cast on this broad distinction by the discovery of some typical Gondwanan vertebrates (traversodont eucynodonts) together with more typical Laurasian elements (phytosaur) in a rich Late Triassic (late Carnian) site near Richmond, Virginia²⁷. On this basis, it was postulated that the apparent variations between north and south were at least in part attributable to age differences. The new finds (in particular *Fraxinopsis* and *Crosaphis*) blur even further the broad distinctions between the Laurasian and

Gondwanan realms. In addition, the new finds call into question some of the perceived age and palaeogeographic differences between the floral assemblages from different parts of North America. □

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A dispersive morph in the naked mole-rat

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CLOSE inbreeding is known for a variety of small mammal species^{1–4} for which a high probability of mortality during dispersal makes helping and delayed maturation a relatively secure fitness option⁵. Prolonged inbreeding, however, is usually associated with lowered fitness^{6,7}, and it has been shown that most highly inbred small mammals⁸ and social insects⁹ have inbreeding-avoidance mechanisms that promote some degree of outbreeding. However, previous field and laboratory research on the naked mole-rat (*Heterocephalus glaber*) suggested that this cooperatively breeding rodent is highly inbred^{10–12}, with new colonies forming by fission¹³. Here we report the discovery of a dispersal phenotype that may occasionally promote outbreeding in naked mole-rats. These dispersers are morphologically, physiologically and behaviourally distinct from other colony members. They are laden with fat, exhibit elevated levels of luteinizing hormone, have a strong urge to disperse, and only solicit matings with non-colony members. These findings suggest that, although rare, a dispersive morph exists within naked mole-rat colonies.

We investigated 48 colonies of captive naked mole-rats

(~1,000 animals) to determine whether outbreeders exist under laboratory conditions. Two critical predictions of the outbreeding hypothesis¹⁴ as applied to naked mole-rats are: (1) some mole-rats should leave their natal burrow system and thus reduce contact with relatives; and (2) the individuals that leave should avoid mating with close kin and should preferentially solicit foreign conspecifics.

To test the first prediction we made a single opening in the burrow system of each colony and recorded which individuals voluntarily left the safe confines of their burrow. Only 6 of the 48 colonies were found to harbour individuals that persistently attempted to disperse (see example in Fig. 1) and, true to the mammalian norm, most of these individuals were male (94.7%, $N = 19$). Interestingly, colonies harbouring dispersers were significantly larger than colonies without dispersers ($\bar{X} = 61.67 \pm 21.3$ ($N = 6$); $\bar{X} = 20.69 \pm 14.32$ ($N = 42$), respectively; $U = -3.67$, $P = 0.0002$). All were well-established colonies (> 4 years), suggesting that, although a genetic basis for dispersal cannot be ruled out, the emergence of dispersers may be triggered by exogenous cues such as the size and age composition of colonies. Furthermore, these individuals, which are above average in size (Fig. 1) and lazy, are likely to impose an energetic burden on the colony, and theoretical predictions¹⁵ suggest that only numerically large colonies could therefore support them.

To test the second prediction of the outbreeding hypothesis we designed a series of mate-choice experiments. Here we provided dispersers and non-dispersers from the same colony with the choice of foreign unrelated versus familiar related conspecifics of both sexes. Importantly, dispersers showed a significant preference ($F_{2,18} = 64.73$, $P < 0.0001$) for unrelated (irrespective of

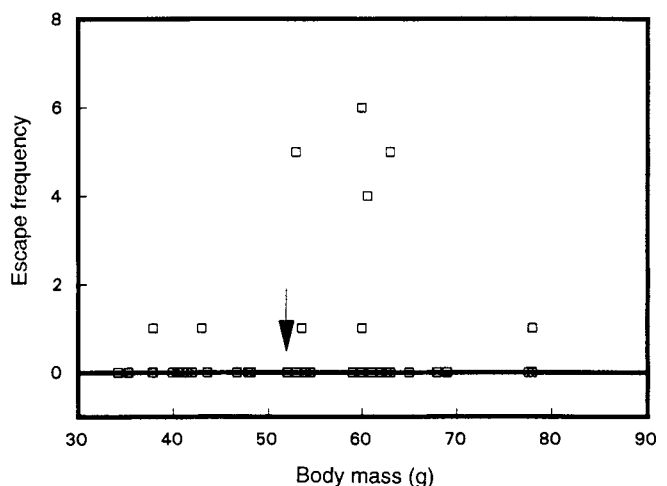
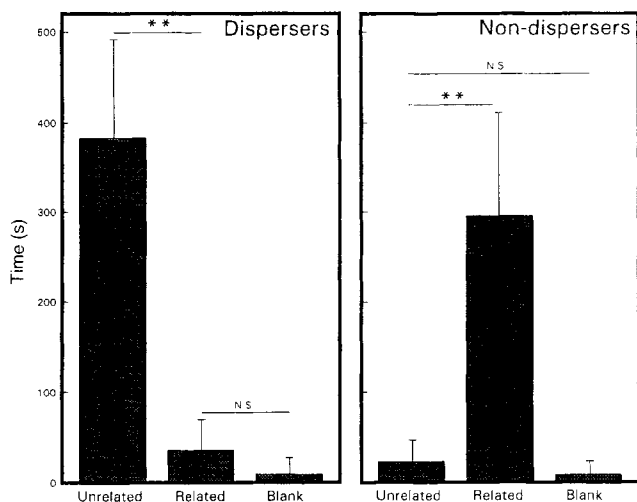


FIG. 1 The number of voluntary attempts to disperse from the natal burrow system, over a period of three consecutive days, for all mole-rats within a single colony of 47 individuals. Individuals that left their burrows were caught in pitfall traps positioned ~30 cm from the opening. The arrow indicates the mean body mass for the colony. Only those individuals that persisted (≥ 3 escapes) in their attempts to leave the natal burrow system were classified as dispersers. Most of the individuals (96%) within our captive population never left their burrows, while only 2.3% left their burrows once, and 1.7% more than three times. To test the response of dispersive versus philopatric mole-rats to a foreign conspecific, we performed a series of triplet associations immediately after the escape trials. In each triplet association we placed a disperser and a non-disperser from the same colony in a circular arena together with a foreign conspecific. All behavioural interactions between the three subjects were recorded for a maximum period of 10 min. In all trials, dispersers showed sexually solicitous behaviour towards the foreign conspecific and attempted to copulate with them ($N = 19$, 100% acceptance). In contrast, non-dispersers always ($N = 7$ trials, 100% rejection) behaved aggressively towards foreign conspecifics. There was no aggressive or sexual behaviour between dispersers and non-dispersers.



sex, $F_{2,10} = 3.02$, $P > 0.05$) versus related mole-rats (Fig. 2). In contrast, as expected from previous studies^{16,17}, non-dispersers were xenophobic and showed a significant preference for colony members ($F_{2,15} = 30.09$, $P < 0.0001$). When placed together with unrelated individuals, dispersers showed solicitous behaviour towards, and attempted to copulate with, the 'foreign' mole-rat. In sharp contrast, non-dispersers from the same colony always responded aggressively to the foreigner.

These results provide the first evidence in support of our hypothesis that outbreeders exist within colonies of naked mole-rats. We then attempted to characterize the phenotype of these highly atypical 'non-breeders' through a variety of physiological, morphological and behavioural measures. These results provide tangible evidence of a distinct dispersive morph that shows some close parallels with the reproductive colony founders of the ants and termites.

The sexually active status of dispersers, as demonstrated by their attempts to copulate with foreign conspecifics, was con-

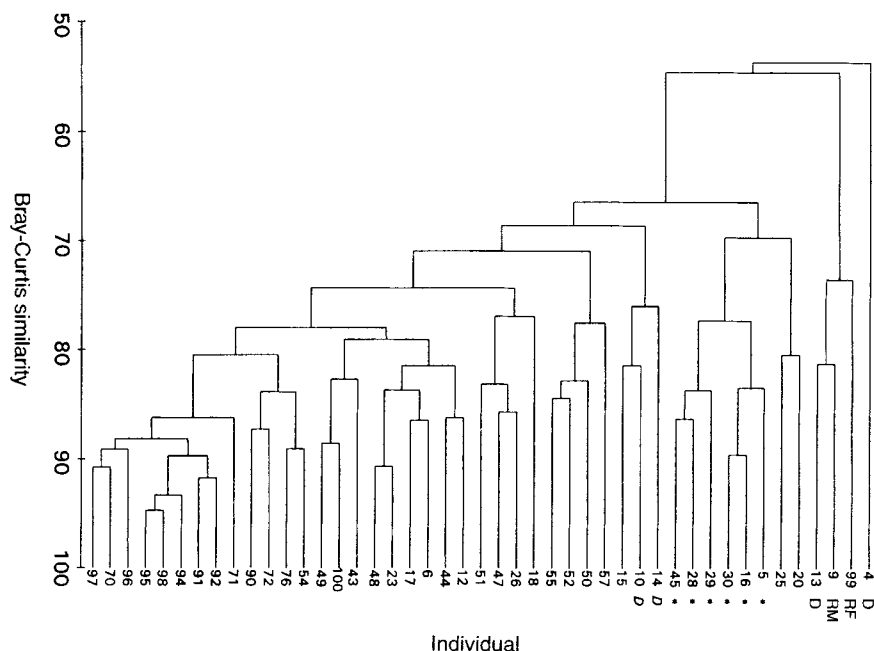
FIG. 2 Results from a three-way choice experiment in which the amount of time (mean $\pm$ s.d.) dispersers ($N = 19$) and non-dispersers ($N = 16$) from the same colony spent attempting to access chambers containing either unrelated or related individuals or an empty chamber. Each trial lasted 10 min, and preference was deduced from the total time spent attempting to access one of the three choice chambers.¹⁷ Test subjects were then allowed access to their preferred chamber and their behavioural interactions with the 'source' individual recorded. Although non-dispersers never showed a preference for foreign conspecifics, their response when placed together with them was also tested ($N = 7$ trials). Each animal was tested once only, to avoid the possible influence of familiarity and learning. The arms of the choice apparatus were rotated in a random fashion between trials to control for any directional bias. The entire apparatus was cleaned between trials. ** $P < 0.0001$; NS, not significant.

firmed physiologically. Dispersers have significantly higher levels of bioactive plasma luteinizing hormone than non-dispersers ($\bar{X} = 8.31 \pm 6.57$, $N = 7$; $\bar{X} = 2.203 \pm 2.09$, $N = 9$ milli-international units per ml, respectively; $U = -1.97$, $P = 0.039$) of a similar age and size, and comparable levels to breeding males ($\bar{X} = 8.28 \pm 3.01$, $N = 2$), indicating that they are sexually primed before departure from their natal burrow.

Behavioural evidence for a unique phenotype was sought through detailed observations of dispersers within one colony of 39 non-dispersers and six dispersers. Dispersers formed a behaviourally distinct group within the colony (Fig. 3). They participated only minimally in cooperative maintenance tasks and displayed a heightened frequency of locomotory and feeding activities when compared to their non-dispersing counterparts. Interestingly, dispersers were not subjected to heightened parental or sibling aggression ($t = -0.15$, $P > 0.05$), despite their selfish disposition, suggesting that they are not forced to leave their natal colony but do so of their own volition. Most importantly, dispersers, despite elevated levels of luteinizing hormone, did not solicit the colony's reproductive female when she was in oestrus, thus fulfilling an essential prediction of the inbreeding-avoidance hypothesis.

Morphological differences were also apparent. Although ske-

FIG. 3 The result of a hierarchical cluster analysis is represented by a dendrogram, with the x-axis defining the similarity level at which any two individuals or groups are considered to be the same (see branches perpendicular to the x-axis), and the y-axis representing the full set of individuals within a single colony of naked mole-rats. The dendrogram was derived from an analysis of the similarities between the behavioural profiles of each individual within a colony of 45 individuals, including the breeding pair. Each number represents a single individual. Numbers denoted with an asterisk have previously been identified as dispersers in escape trials. RF denotes the reproductive female, RM the reproductive male, and D defenders. Cluster analysis aims to find 'natural groupings' of individuals such that individuals within a group are more similar to each other than to individuals in different groups. It is clearly evident that dispersers form one such group and are thus more similar to one another ($> 75\%$) than to any other colony members. This was confirmed statistically with an analysis of similarity²⁰ ($R_{1,44} = 0.45$, $P = 0.001$). Behavioural profiles were compiled from 1,200 behavioural acts per individual recorded in 40 h of 2-min-interval scan sampling observations. We included 35 behaviours in the raw data matrix, including all work, interactive, agonistic, locomotory and feeding-related activities (*sensu* Lacey and Sherman¹⁵). Before all analyses the data were root-root transformed to avoid biasing the results in favour of those behaviours performed at either very low or high



frequencies. The Bray-Curtis similarity coefficient²⁰ was chosen because it is not affected by joint absences in the data (that is, when individuals never perform a given behaviour), an inherent property of the behavioural profiles of individuals within a naked mole-rat colony.

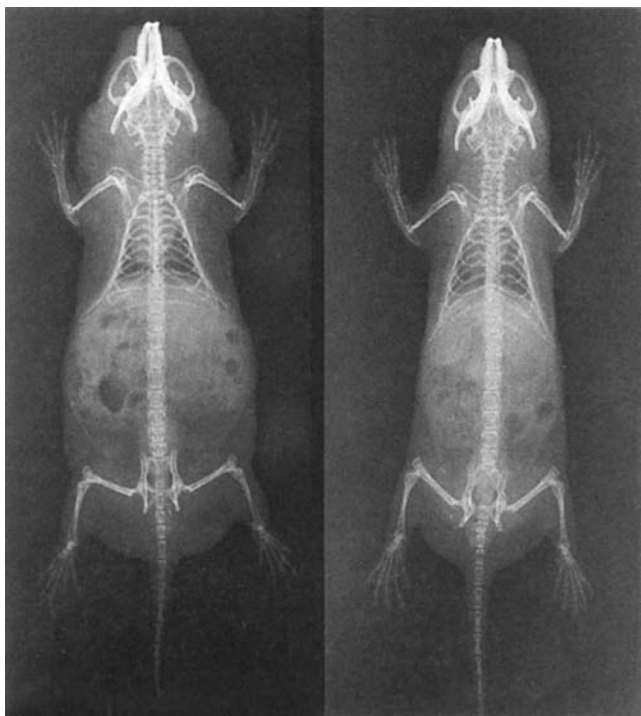


FIG. 4 X-ray images of a disperser (left) and a non-disperser (siblings) naked mole-rat provide evidence for a distinct phenotype. Whereas dispersers ($X \pm s.d. = 56.57 \pm 11.45$) were significantly heavier ($t = 2.889$, $P = 0.016$) than a random sample of non-dispersers ($X \pm s.d. = 40.67 \pm 5.57$) from the same age cohort, there was no significant difference in the skeletal robustness of the two groups. This mass difference reflects the significantly higher percentage body fat of dispersers (two-tailed t -test, $t = 2.58$, $P = 0.02$) as determined from electromagnetic scanner analyses. As a consequence, dispersers had a significantly higher girth-to-body-length ratio (two-tailed t -test, $t = 6.04$, $P = 0.0006$), and more fat in the neck region; both features are evident in the soft-tissue outlines of the X-rays. In contrast, colony defenders of a comparable body mass had little subcutaneous fat.

letal variables did not differ significantly between dispersers and non-dispersers from the same colony, dispersers had significantly greater total body fat than non-dispersers of a similar body mass and age (Fig. 4). The larger fat reserves in dispersers may serve as a nutritional safeguard against starvation during dispersal and colony founding, in a manner similar to the fatty tissues of the winged alates of termites¹⁸.

The paucity of outbreeding events in wild naked mole-rats may reflect the high somatic fitness costs (for example, burrowing and predation) associated with dispersal¹⁹ for these small, subterranean mammals. Furthermore, members of a colony are typically xenophobic towards foreign conspecifics^{16,17}, posing a threat to successful dispersers. However, both laboratory ($N = 3$) and field ($N = 1$; S. Braude, personal communication) evidence has shown that mole-rats are occasionally accepted into foreign colonies and may successfully outbreed. In all three captive colonies the breeding male had died before the arrival of the dispersers. Crucial to our hypothesis, two of these three captive 'dispersers' became reproductives in the foreign colony, despite the presence of natal adult males (M.J.O.R. and J.U.M.J., unpublished data). In one of these examples the disperser has maintained breeder status for over two years.

We do not know when and how dispersers leave their natal colony to locate foreign mole-rats, but suggest that, occasionally, favourable environmental conditions may promote dispersal events in which this specialized dispersive morph may successfully outbreed by either invading established colonies or by soliciting a few foreign individuals to form a nascent colony. □

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What is rivalling during binocular rivalry?

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WHEN different images are presented to the two eyes, they compete for perceptual dominance, such that one image is visible while the other is suppressed. This binocular rivalry is thought to reflect competition between monocular neurons within the primary visual cortex¹. However, neurons whose activity correlates with perception during rivalry are found mainly in higher cortical areas, and respond to input from both eyes^{2,3}. Thus rivalry may involve competition between alternative perceptual interpretations at a higher level of analysis. To investigate this, we tested the effect of rapidly alternating the rival stimuli between the two eyes. Under these conditions, the perceptual alternations exhibit the same temporal dynamics as with static patterns, and a single phase of perceptual dominance can span multiple alternations of the stimuli. Thus neural representations of the two stimuli compete for visual awareness independently of the eye through which they reach the higher visual areas. This finding places binocular rivalry in the general category of multistable phenomena, such as ambiguous figures, and provides a new way to study the neural cause and resolution of perceptual ambiguities.

Experiments showing that test probes, bearing no resemblance to a suppressed stimulus, are more difficult to detect when presented to an eye during suppression than during dominance, suggest that suppression operates non-selectively over a broad range of stimuli, involving the suppression of the eye rather than the suppression of a particular stimulus^{4–7}. Eye competition is also suggested by the observation that immediately after a subject reports exclusive suppression of one eye's stimulus, this stimulus becomes instantly visible when it is rerouted into the already

dominating eye⁸. However, when targets of different colour and structure are presented dichoptically (to the two eyes), the structural information from one monocular image can combine with the colour information from the other eye^{9,10}. In addition, when such stimuli are composed of multiple parts, then those parts that form a Gestalt figure, or simply have the same colour, are more likely to dominate together perceptually, even if they are presented to opposite eyes^{11,12}. Such findings suggest that there is selective suppression of different parts or aspects of the two monocular stimuli, and favour the idea of competition between stimulus representations, which is supported by physiological data showing that, in non-human primates, the activity of binocular rather than monocular neurons correlates with perception during competition between the eyes^{2,3}.

To test the merits of these two views, we used a new stimulus paradigm in which the rivaling patterns were periodically exchanged between the two eyes, thereby eliminating the possibility that perceptual alternations occur simply as a result of fatigue of one or the other oriented monocular channel (Fig. 1). Thus 'eye suppression' predicts periods in which perception is

dominated by a grating that regularly switches orientation, as would be seen if the subject closed one eye. However, 'stimulus suppression' predicts normal rivalry between the two continually exchanged patterns, because the conflicting central representations of these patterns remain the same, regardless of the eye through which they come.

Six observers, including the three authors, who have normal or corrected vision and normal stereopsis, participated in these experiments. The observers were tested with sinusoidal gratings of orthogonal orientations that were flickered on and off with a frequency of 18 Hz (Fig. 1). Two types of trials were used: (1) non-reversal trials, in which the orientations of the stimuli remained unchanged in each eye throughout the trial (control condition); and (2) reversal trials, in which the flickering gratings were exchanged between the two eyes every 333 ms. The selected reversal time was long enough to preclude abnormal fusion, but less than the time required for newly presented rivalrous stimuli to yield exclusive visibility of one stimulus¹³. Although other swap

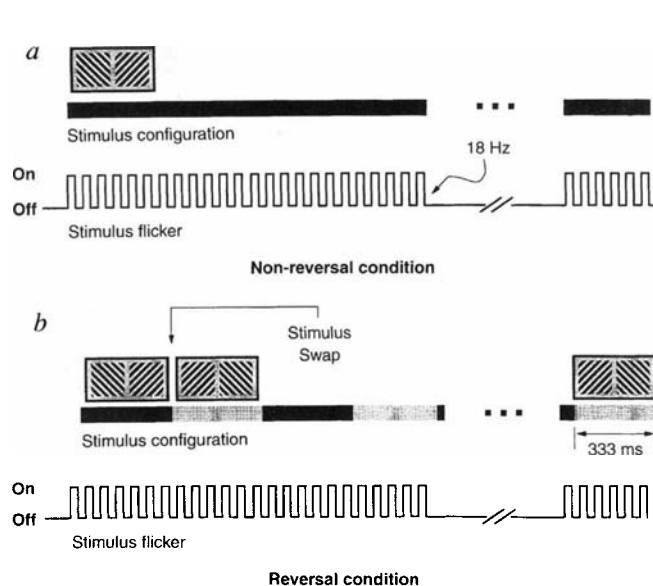


FIG. 1 During an experimental session the observer viewed a pair of orthogonally oriented gratings (shown in the insets) rightward tilted (45°) to one eye and leftward (-45°) to the other. *a*, Time course of a non-reversal trial. The same stimuli (for example, right orientation in the right and left orientation in the left eye) were flickered synchronously in each eye at 18 Hz (flashes of 27.7 ms duration that were separated by intervals of 27.7 ms). *b*, Time course of a reversal trial, in which the flickering gratings were exchanged between the eyes every 333 ms. In this condition, each eye was presented with a grating that periodically switched between perpendicular orientations. The stimulus configuration bar shows the stimulus-to-eye correspondence at any given time.

METHODS. The observers viewed the stimulus displays through a mirror stereoscope. The stimuli were generated by a Silicon Graphics computer (Indigo/Elan, 72 frames s^{-1}), and displayed on a 21-inch Sony monitor located 60 cm from the eyes of the subject. Stimuli were square patches ($3^\circ \times 3^\circ$) of sinusoidal gratings. The contrast of the gratings was 20%, their spatial frequency 2.5 cycles per degree, and their space average luminance was 20.5 cd m^{-2} . The background luminance was 1.5 cd m^{-2} . The contrast between the mean luminance of the grating and that of the background, together with a small ($0.2^\circ \times 0.2^\circ$) fixation spot were used to aid proper convergence. Care was taken in adjusting the mirrors of the stereoscope to ensure correct binocular alignment of the displays for each observer. Each observer participated in 9 sessions of 13 trials, with each trial lasting 130 s, and 3 sessions devoted to each stimulus condition. A session started with a few minutes of practice trials and continued for 75–80 min, with 5–10 min rest every 20 min. The subjects reported exclusive perceptual dominance of each stimulus orientation by holding down buttons on a computer mouse. The subject was illustrated to press neither button during periods of piece-meal rivalry. Data analysis was restricted to the last 120 s of each trial.

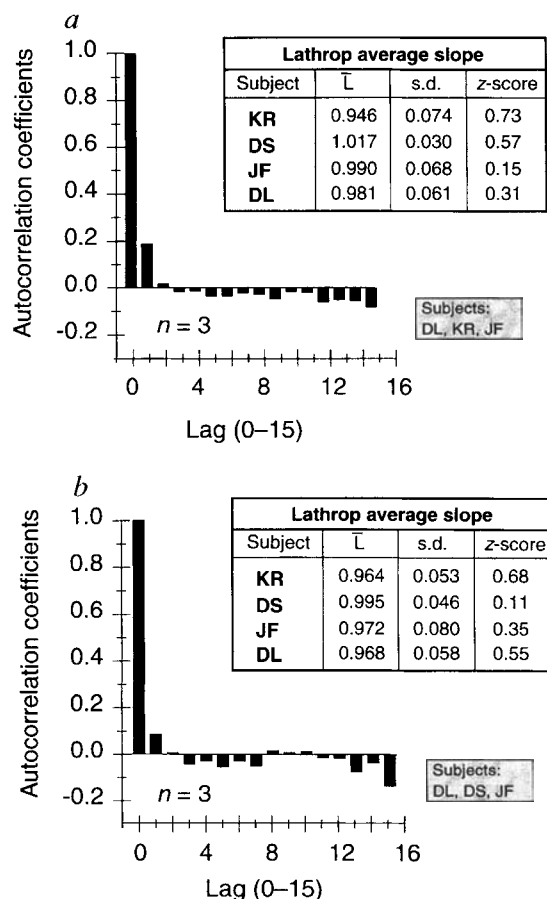


FIG. 2 Temporal dynamics of rivalry under different stimulus conditions. Autocorrelation coefficients for sequences of rivalry phases, averaged for three different subjects. *a*, Data collected in the non-reversal trials; *b*, data collected in the reversal trials. Note the very low correlation coefficients in both conditions for all but the 'trivial' zero lag. The tables next to each plot give the values of the Lathrop statistic, L (ref. 17). The value of L is a measure of the mean absolute slope of successive durations given by the formula $L_j = \sqrt{\{\sum_{i=1}^{N-1} |x_{i+1,j} - x_{i,j}| / [(N-1)\sigma_j]\}}$, where L_j equals the value of the statistic for the j th sequence (here all phases recorded from a subject; $x_{i,j}$, $x_{i+1,j}$ are successive responses in the j th sequence; and σ equals the standard deviation of the phases within the j th sequence. The theoretical L_j value distribution has a mean $\bar{L} = 1.0$ and a $\sigma_L = 1/(2\sqrt{N})$. A correlation between successive phases yields L_j values that are lower, and an anticorrelation yields L_j values that are greater than 1.0. Note that none of the obtained values, in either of the paradigms, differs significantly (Z-scores) from the expected value of $\bar{L}$ (1.0 for random sequences).

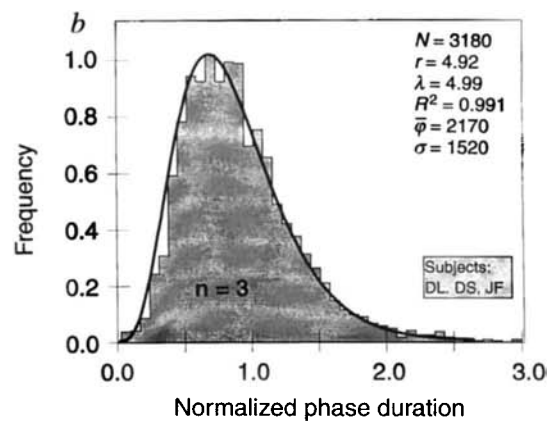
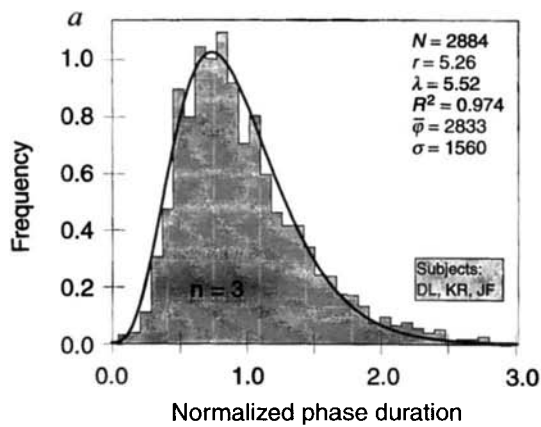


FIG. 3 Distribution of dominance of rivalry in *a*, non-reversal and *b*, reversal conditions. The frequency histograms show the distribution of relative phase durations, that is, phase durations expressed as a fraction of the mean phase duration. The smooth, thick, black lines illustrate the approximation of the data with a gamma distribution $f(x) = \lambda^r / \Gamma(r) x^{r-1} \exp(-\lambda x)$, where $\Gamma(r) = (r-1)!$. The parameters of the theoretical distribution describing the data obtained during either condition are very similar. The value n shows the number of subjects

whose data were used to create the frequency distributions; N denotes the number of phases recorded from all subjects; R^2 is the coefficient of determination, that is, the square of the correlation between the experimental data and the corresponding values of the gamma function (R^2 equals the ratio of the explained sum of squares of the frequency variable to the total sum of squares of this variable); $\bar{\phi}$ denotes the mean phase duration in ms across all subjects; and σ the standard deviation of the phases.

times (for example, 222 or 444 ms) gave similar results, the selected time proved to be the most effective for all subjects. The stimuli were flickered to minimize or eliminate the awareness of instances of stimulus reversals. Testing without flicker yielded similar results.

In both conditions, all six subjects reported normal rivalry with phases of complete dominance of a stimulus persisting for up to several seconds. The mean dominance time for all subjects in the reversal trials was 2,350 ms, spanning on average over seven stimulus exchanges. To establish whether the perceptual rivalry experienced in the reversal trials is the same as that experienced with conventional stimuli, we examined the temporal dynamics of the perceptual alternations. Specifically, we examined: (1) the extent to which rivalry phases are sequentially independent by performing an autocorrelation analysis of successive durations and calculating a statistic that tests sequential dependence¹⁴; (2) the distribution of alternation phases; and (3) the influence of the contrast of one stimulus on the mean dominance duration of the other^{15,16}.

The product-moment correlation coefficients that were calcu-

lated for the durations of dominance and suppression through lag 15 are shown in Fig. 2. Data from three subjects are shown for rivalry recorded in non-reversal (Fig. 2*a*) and reversal (Fig. 2*b*) trials. Note that, even for the shortest lag, the durations of rivalry are stochastically independent in both experiments, just as reported for conventional rivalry¹⁴. Absence of sequential dependence was also shown by using the Lathrop statistic^{14,17}, a measure of the mean absolute slope of successive durations for each subject (Fig. 2).

The distribution of relative phase durations (durations expressed as fractions of their mean) during standard binocular rivalry is typically approximated by a gamma density function, the parameters of which show consistent intersubject similarity^{3,14,16,18}. The distributions of relative phases for the non-reversal and reversal conditions are shown in Fig. 3. In agreement with previous studies, both distributions were found to be unimodal, asymmetric with a fast growth and a long tail, and both were approximated well with gamma distributions having very similar parameters.

Finally, the effects of the strength of one stimulus on the mean

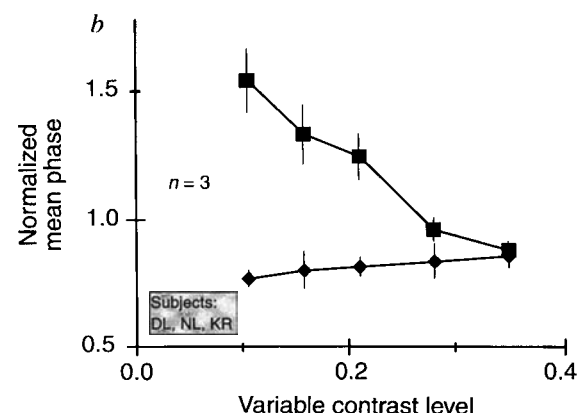
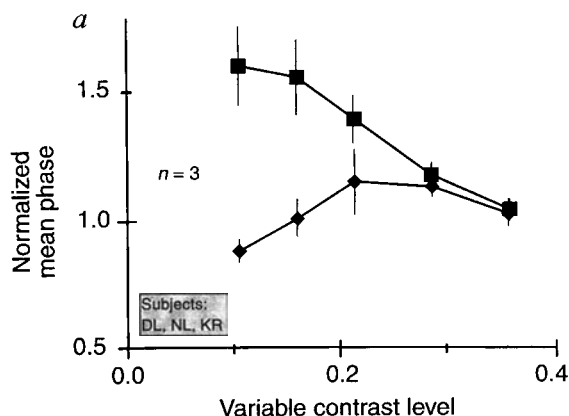


FIG. 4 Effects of variation of interstimulus contrast on the mean dominance phase. Fixed contrast was set to 0.35. On the abscissa is plotted the varied contrast of one stimulus, when the stimulus is presented always in the same eye (*a*), and when the stimulus is alternating between the left and right eyes (*b*). The ordinate shows the normalized mean dominance duration of the stimulus whose contrast is varied systematically

(diamonds), and of the stimulus that has fixed contrast (squares). Data are averaged from three subjects. The bars show the standard error of the mean. Note that, in both conditions, decreasing the strength (here contrast) of one stimulus primarily results in a monotonic increase in the mean dominance time of the fixed-contrast stimulus.

dominance and suppression of each stimulus were examined. During conventional rivalry, increasing the stimulus strength in one eye causes a change in the mean duration of dominance of the contralateral eye, but the mean duration of the dominance of the changed stimulus is only minimally affected^{15,16,19}. A similar pattern for both the non-reversal and the reversal trials is shown in Fig. 4. Note that in the reversal trials, the contrast of one orientation primarily affects the mean dominance time of the other orientation, even though each eye sequentially sees both the fixed- and variable-contrast stimuli. Hence it is the strength of the competing stimuli that governs this effect.

Taken together, these results show that the rivalry experienced when monocular stimuli are continually swapped between the eyes is indistinguishable from conventional binocular rivalry, based on the standard tests of the temporal dynamics of this phenomenon. The smooth, slow perceptual changes, despite continuous alternation of the stimulus orientation in each eye, exclude the possibility that rivalry is the result of complete suppression of one monocular channel. Each monocular view is a grating that periodically changes orientation as the stimuli are exchanged between the eyes. Yet these physical changes of the monocular view are rarely, if ever, visible, and perception is instead dominated for extended periods by one or the other orientation, with the left- and right-tilted grating patterns alternating in conscious perception slowly and stochastically. The effect reported here appears to vary in strength as the contrast or the type of the stimulus changes. For two observers, increasing the contrast of the stimulus disrupted 'stimulus-rivalry', and perception was dominated by a mixed stimulus, parts of which often followed the physical monocular orientation changes. However, using a red right-tilted and a blue left-tilted grating as rivaling stimuli yielded the best exclusive 'stimulus rivalry'. Note that in the 'reversal' trials, monocular perception can be the oscillatory apparent rotation-motion of a single achromatic grating, which is a third possible stimulus interpretation. The addition of an orientation-specific colour in the stimuli may eliminate any perception of apparent motion, resulting in even stronger perceptual bistability than experienced with achromatic stimuli.

In conclusion, we suggest that it is the 'stimulus' and not the 'eye' that competes for dominance during rivalry; dichoptic stimulation provides only a convenient means to present two conflicting stimuli simultaneously. This conclusion does not preclude local inhibitory interactions from contributing to the singleness of binocular vision. Such interactions are thought to be essential in stereopsis²⁰, and may also contribute in the instability observed during rivalry; however, they are unlikely to be the basis of exclusive dominance of one stimulus pattern. Instead, perceptual alternations during dichoptic stimulation are most likely to be the result of perturbations of the same neural machinery involved in the cause and resolution of other multistable perceptual phenomena, such as monocular rivalry²¹ and ambiguous figures, which show similar dynamics to binocular rivalry²². Thus rivalry, in which arbitrary combinations of conflicting stimuli battle for dominance at a central stage of processing, provides an excellent tool with which to study spatial and form vision, both psychophysically and physiologically. □

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Inhibition of an inwardly rectifying K⁺ channel by G-protein α -subunits

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CHOLINERGIC muscarinic, serotonergic, opioid and several other G-protein-coupled neurotransmitter receptors activate inwardly rectifying K⁺ channels of the GIRK family, slowing the heartbeat and decreasing the excitability of neuronal cells¹. Inhibitory modulation of GIRKs by G-protein-coupled receptors may have important implications in cardiac and brain physiology. Previously G_α and G_{βγ} subunits of heterotrimeric G proteins have both been implicated in channel opening^{2,3}, but recent studies attribute this role primarily to the G_{βγ} dimer that activates GIRKs in a membrane-delimited fashion, probably by direct binding to the channel protein^{4–8}. We report here that free GTPγS-activated G_{o1}, but not G_{o2} or G_{o3}, potently inhibits G_{β1γ2}-induced GIRK activity in excised membrane patches of *Xenopus* oocytes expressing GIRK1. High-affinity but partial inhibition is produced by G_{as}-GTPγS. G_{o1}-GTPγS also inhibits G_{β1γ2}-activated GIRK in atrial myocytes. Antagonistic interactions between G_α and G_{βγ} may be among the mechanisms determining specificity of G protein coupling to GIRKs.

Activation of heterotrimeric G proteins involves GDP–GTP exchange at the G_α subunit followed by dissociation of the G_{αβγ} complex into free (activated) G_{βγ} and G_α-GTP⁹. Various combinations of G_β and G_γ subtypes activate atrial GIRK with similar potency⁵; it is therefore puzzling that activation of GIRK in atrial and neuronal cells is normally coupled only to pertussis-toxin-sensitive G proteins (G_i and possibly G_o subclasses)^{10–12}, rather than to all neurotransmitter-activated G proteins¹³. We tested for possible synergistic or antagonistic interactions between G_α and G_{βγ} that could in part account for the specificity of G protein/GIRK coupling¹⁴.

In excised inside-out membrane patches of *Xenopus* oocytes expressing a cardiac/brain GIRK subunit^{15,16} (GIRK1 (KGA)), purified recombinant G_{β1γ2} activated a ~40 pS inwardly rectifying K⁺ channel absent in native oocytes (Figs 1a and 2a, and data not shown; this channel probably represents a heteromultimer of GIRK1 and an endogenous oocyte's subunit, XIR¹⁷). Full activity developed within 2–3 min and remained constant for >10 min

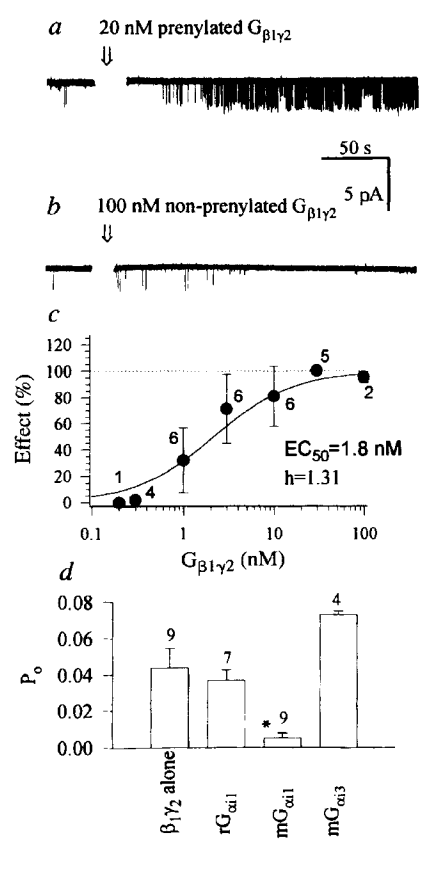
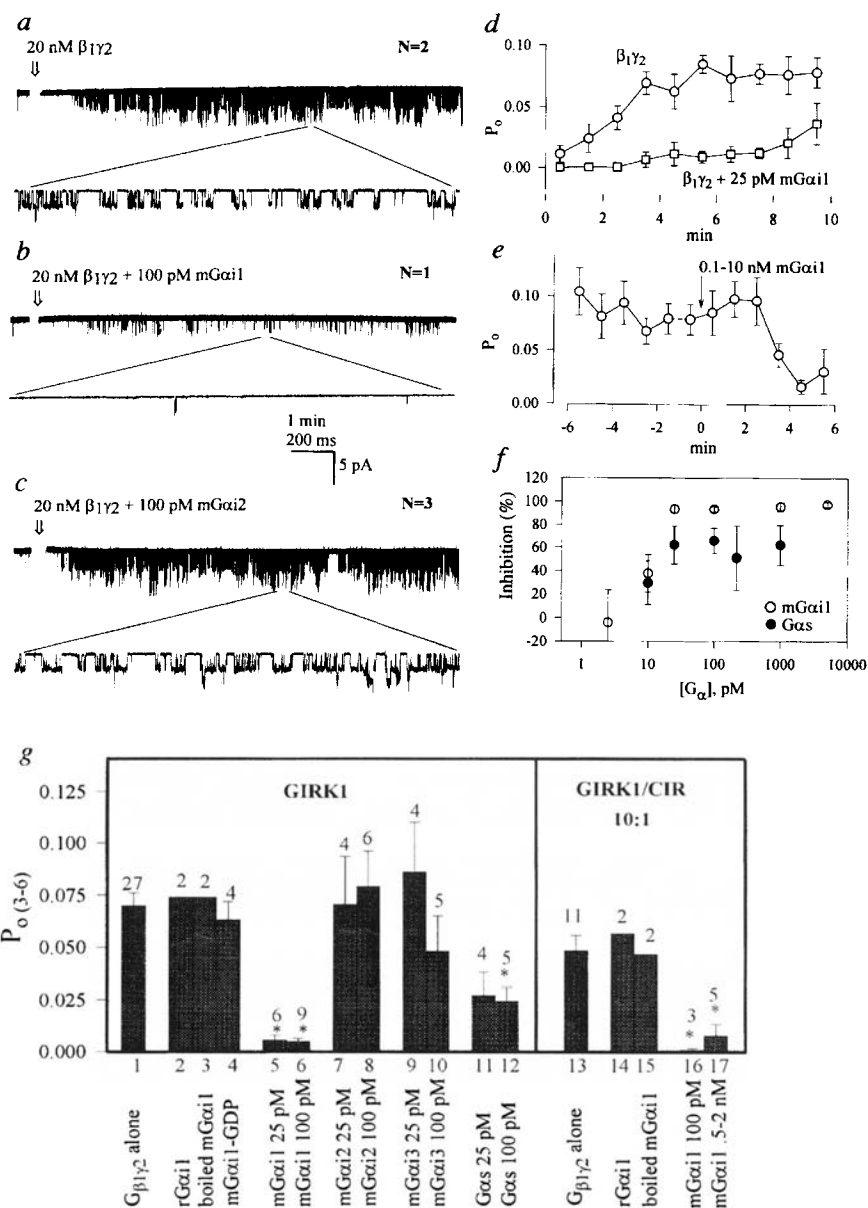


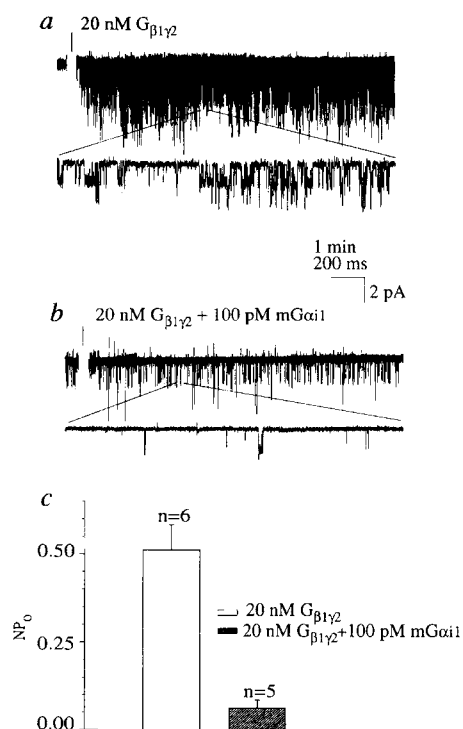
FIG. 2 $mG_{\alpha 1}$ -GTP- γ S and $mG_{\alpha 3}$ -GTP- γ S inhibit GIRK. **a–c**, $G_{\beta 1 \gamma 2}$ activation of GIRK1 (**a**) is suppressed by coapplication of 100 pM $mG_{\alpha 1}$ -GTP- γ S (**b**) but not $mG_{\alpha 3}$ -GTP- γ S (**c**). **N**, Number of channels in the patch. **d**, Kinetics of GIRK1 activation by 20 nM $G_{\beta 1 \gamma 2}$ alone ($\circ$; $n = 8$) or with 25 pM $mG_{\alpha 1}$ -GTP- γ S ($\square$; $n = 4$). **e**, Effect of addition of $mG_{\alpha 1}$ -GTP- γ S (at $t = 0$) 10–13 min after activation of GIRK1 by 20 nM $G_{\beta 1 \gamma 2}$ ($n = 5$). **f**, Dose-dependency of GIRK1 inhibition by $mG_{\alpha 1}$ -GTP- γ S ($\circ$) and $G_{\alpha 3}$ -GTP- γ S ($\bullet$). Data points show mean $\pm$ s.e.m. in 3–9 cells. The average value of P_o between minutes 3 to 6 after $\beta_1\gamma_2$ addition, $P_{o(3-6)}$, was used to access channel activity. **g**, Summary of effects of coapplication of various G_x proteins on $G_{\beta 1 \gamma 2}$ -induced GIRK activity. Bars show mean $P_{o(3-6)} \pm$ s.e.m., with number of patches indicated. Asterisks denote statistically significant difference ($P < 0.05$). Groups 5–12 were compared with group 1, and groups 16 and 17 with group 13, using one-way ANOVA test followed by Bonferroni t -test.

METHODS. The oocytes were injected with GIRK1 RNA alone or 0.1–1 ng GIRK + 0.01–0.1 ng CIR (rcKATP²⁹) RNAs (GIRK1/CIR 10:1). Recombinant myristoylated and non-myristoylated G_x proteins were prepared as described³⁰ (note that $G_{\alpha s}$ was not palmitoylated), activated by incubation with 50 mM Na-HEPES (pH 8.0), 10 mM MgSO₄, 1 mM EGTA, 2 mM DTT and 400 μ M GTP- γ S for 30 min ($G_{\alpha s}$ and $G_{\alpha o}$) or 2 h ($G_{\alpha 1}$). Free GTP- γ S was removed by gel filtration in G_x buffer (20 mM Na-HEPES (pH 8.0), 2 mM MgSO₄, 1 mM EDTA, 2 mM DTT) and the proteins were stored in this buffer in 2–10- μ l aliquots at 2.5–20 μ M at -80°C ³⁰. An aliquot was thawed at 30°C , placed on ice, diluted in G_x buffer if necessary, then applied to the bath as described for $G_{\beta 1 \gamma 2}$ in Fig. 1 (except that the concentration of ATP was 2 mM in these experiments) no later than 5 min after thawing.

FIG. 1 Activation of GIRK1 by $G_{\beta 1 \gamma 2}$ in excised inside-out patches at -80 mV, and inhibition by pre-exposure to $mG_{\alpha 1}$ -GTP- γ S. Prenylated $G_{\beta 1 \gamma 2}$ (**a**), but not non-prenylated $G_{\beta 1 \gamma 2}$ (**b**), activates GIRK1. The record during $G_{\beta 1 \gamma 2}$ addition to the bath was blanked out. **c**, Dose-dependency of activation of GIRK1 by prenylated $G_{\beta 1 \gamma 2}$. Data points indicate mean $\pm$ s.d. and number of patches. Only patches containing ≤ 4 channels (estimated from maximal number of overlaps) were analysed. $G_{\beta 1 \gamma 2}$ concentrations were applied to a patch sequentially in ascending order, for 10 min each, and the single channel open probability, P_o , was calculated from idealized current record in 5-s bins, averaged between 3 and 10 min after $G_{\beta 1 \gamma 2}$ addition, and normalized to the highest P_o observed in that patch (usually at 30 nM $G_{\beta 1 \gamma 2}$). Data were fitted to the equation: $f = 100(1 - 1/(1 + 10^{h(\log[C] - \log[EC_{50}])))$, where f is the fractional response, C is $G_{\beta 1 \gamma 2}$ concentration, EC_{50} is the concentration producing half maximal effect, and h is the Hill coefficient. **d**, Effects of pre-exposure to $mG_{\alpha 1}$ -GTP- γ S, $rG_{\alpha 1}$ -GTP- γ S or $mG_{\alpha 3}$ -GTP- γ S on GIRK1 activation by $G_{\beta 1 \gamma 2}$.

METHODS. Oocytes were isolated and injected as described²⁷ with 0.1–1 ng GIRK1 RNA alone or with 0.1–0.5 ng RNA of human m2 receptor, m2R¹⁶. Single-channel currents (at -80 or -60 mV) were filtered at 2 kHz, sampled at 5 kHz and analysed as described¹⁶. Pipette solution contained (in mM) 144 KCl, 2 NaCl, 1 MgCl₂, 1 CaCl₂, 0.05–0.1 GdCl₃, 10 HEPES/KOH, pH 7.5. Bath (500 μ l) solution contained, in mM: 140 KCl, 6 NaCl, 4 MgCl₂, 1 EGTA, 1 Na-ATP, 0.1 GDP- β S, 10 HEPES/KOH, pH 7.5. Wild-type (prenylated) and mutant (non-prenylated: $G_{\beta 1 \gamma 2}$ C68S) $G_{\beta 1 \gamma 2}$ were prepared as described^{18,28} and stored in 3–5- μ l aliquots at -80°C in $G_{\beta 1 \gamma 2}$ storage buffer containing 0.7% CHAPS²⁸. Two to eight min after patch excision, 0.5–2 μ l $G_{\beta 1 \gamma 2}$ stock solution was diluted into 50 μ l bath solution which was added to the bath and mixed. G_x subunits were prepared and treated as described in Fig. 2 legend.





(Fig. 2a, d). Neither $G_{\beta\gamma}$ storage buffer ($n > 10$; data not shown), nor $G_{\beta 1\gamma 2}$ containing a non-prenylated mutant of $G_{\beta 2}$ ($G_{\beta 2C68S}$) that does not modulate adenyl cyclase types I or II¹⁸, activated the channel (Fig. 1b; $n = 11$). Activation by $G_{\beta 1\gamma 2}$ displayed a half-maximal effective concentration (EC_{50}) of ~ 2 nM and was maximal at ≥ 30 nM (Fig. 1c). In bath solution containing 0.1 mM GDP- β S (to prevent channel activation by trace amounts of GTP-

FIG. 3 mG α_{i1} -GTP- γ S inhibits GIRK in atrial myocytes. a, b, Examples of channel activity records in inside-out patches after addition of $G_{\beta 1\gamma 2}$ alone (a) or together with 100 pM mG α_{i1} -GTP- γ S (b). c, Summary of the experiments in atrial myocytes. Total open probability of all channels present in the patch, NP_o , rather than P_o , was used for comparison purposes, because myocyte patches usually contained more than 4 channels, and because the strong inhibitory effect on mG α_{i1} led to an underestimation of N in mG α_{i1} -treated patches. Bars show mean $\pm$ s.e.m.

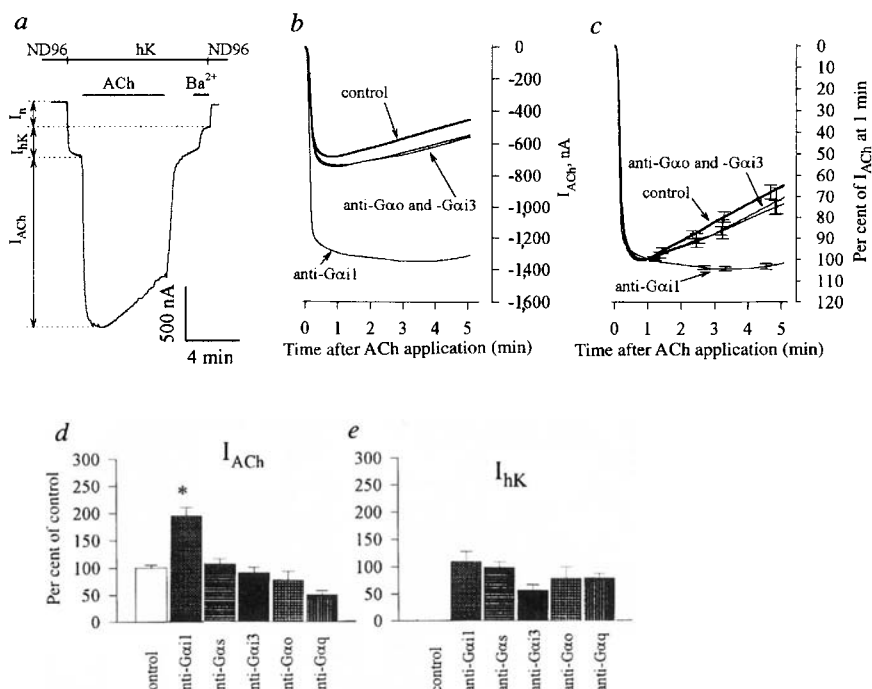
METHODS. Atrial cells from adult rats were isolated as described¹⁶. Patch-clamp experiments were performed 1–4 days after cell disaggregation at 20 °C, using the same solutions as in oocyte experiments, but without CdCl₃ in the pipette solution. Patch pipettes had resistances between 18 and 19 M Ω , and patches were estimated to contain 5–6 channels. Currents were filtered at 2 kHz and digitized at 4 kHz. Data acquisition and analysis were done as described in the legend to Fig. 1.

γ S that could be released from the purified G_{α})⁵, myristoylated recombinant GTP γ S-activated $G_{\alpha i1}$ or $G_{\alpha i3}$ (mG α_{i1} -GTP γ S; mG α_{i3} -GTP γ S; 2–20 nM) did not activate GIRK1 (data not shown; refs 5 and 6). However, a 10-min pre-exposure of the patches to 10–20 nM mG α_{i1} -GTP γ S, but not mG α_{i3} -GTP γ S or non-myristoylated $G_{\alpha i1}$ -GTP γ S (rG α_{i1} -GTP γ S), suppressed $G_{\beta 1\gamma 2}$ activation of the channel (Fig. 1d).

To mimic the physiological situation, $G_{\beta 1\gamma 2}$ was added to the bath simultaneously with G_{α} subunits. Unexpectedly, coapplication of mG α_{i1} -GTP γ S at concentrations as low as 25–100 pM almost completely prevented activation by 20 nM $G_{\beta 1\gamma 2}$ (Fig. 2b). Half-maximal inhibition occurred at ~ 10 pM mG α_{i1} -GTP γ S (Fig. 2f). mG α_{i2} -GTP γ S (100 pM) had no inhibitory effect (Fig. 2c). Partial relief of mG α_{i1} -GTP γ S inhibition was observed after 7–10 min (Fig. 2d), perhaps because of instability of the $G_{\alpha i1}$ protein, or because of a feedback mechanism effected by mG α_{i1} -GTP γ S or $G_{\beta 1\gamma 2}$. When mG α_{i1} -GTP γ S was applied after the channel was already fully activated by $G_{\beta 1\gamma 2}$, the inhibitory effect developed with a delay of 2–3 min and was more variable than upon coaddition with $G_{\beta 1\gamma 2}$ (Fig. 2e).

FIG. 4 Antisense oligodeoxynucleotide (ODN) effects on whole-cell GIRK1 current. a, Membrane currents recorded at -80 mV in an oocyte-expressing GIRK1 and m2R. Solutions and two-electrode voltage clamp were as described¹⁶. Replacement of low- K^+ (ND96; 2 mM K^+) solution with high- K^+ (hK; 96 mM K^+)¹⁶ solution elicits an inward current through endogenous non-GIRK channels (I_n) insensitive to block by Ba^{2+} (300 μ M), and through basally active GIRK1 (I_{hK}). ACh (10 μ M) evokes K^+ current through GIRK1 channels, I_{ACh} ¹⁶. b, Effects of anti- $G_{\alpha i1}$, - $G_{\alpha i3}$ and - $G_{\alpha o}$ ODNs on I_{ACh} . Traces represent average I_{ACh} in 4–5 oocytes from a single donor. c, Comparison of kinetics of currents shown in b. In each oocyte, I_{ACh} was normalized to the amplitude measured 1 min after ACh addition; normalized currents were averaged (representative s.e.m.s are shown). d, e, Summary of the effects of ODNs on the amplitude of I_{ACh} and I_{hK} , respectively, in 2–4 experiments like that shown in b. In each oocyte, I_{ACh} and I_{hK} were expressed as per cent of the mean amplitudes of these currents in the control group of oocytes from the same donor. These normalized values were averaged across all oocyte batches tested. Statistical analysis as in Fig. 2g.

METHODS. Oocytes were injected with RNAs encoding GIRK1 (200–350 pg) and m2R (20 pg), incubated for 2–4 days, then injected with 30 nl of 1 ng ml⁻¹ ODNs, and incubated for an additional 45–55 h. ODN design included 5' and 3' end capping of 2 and 4 phosphorothioates (PSs), respectively, and a



PS at every third internal position to enhance nuclease resistance. Oocytes of the control group were injected with a mismatched PS-ODN of the following sequences: ATCGTTGTGAGCGCTTCGGCATCGGT.

In the atrium, the $I_{K_{ACh}}$ channel is probably a heteromultimer of GIRK1 and an additional subunit (CIR¹⁹ (GIRK4, Kir3.4²⁰)) whose coexpression with GIRK1 in *Xenopus* oocytes strongly enhances whole-cell GIRK currents^{19,21}. The effect of $mG_{\alpha 1}$ -GTP γ S was independent of CIR; it produced similar inhibition in oocytes injected with GIRK1 alone or with GIRK1 and CIR RNAs at 10:1 or 1:10 ratio (Fig. 2g, and data not shown). Inhibition by $mG_{\alpha 1}$ -GTP γ S was not a peculiarity of the oocyte expression system: in rat atrial myocytes, $G_{\beta 1,2}$ -induced activity of the $I_{K_{ACh}}$ channel (GIRK1/CIR) was also strongly inhibited by 100 pM $mG_{\alpha 1}$ -GTP γ S (Fig. 3).

Similar to $mG_{\alpha 1}$ -GTP γ S, $mG_{\alpha 3}$ -GTP γ S had little or no inhibitory effect on GIRK1 activation (Fig. 2g), whereas $mG_{\alpha 2}$ -GTP γ S produced variable results, with an average of about 50% inhibition at 25 and 100 pM (data not shown). $G_{\alpha s}$ -GTP γ S was the only other G_{α} subunit studied that reproducibly inhibited GIRK1 (Fig. 2g). $G_{\alpha s}$ -GTP γ S has an EC_{50} similar to $mG_{\alpha 1}$ -GTP γ S (Fig. 2f) but a lower efficacy (maximal inhibition was ~60%), implying that $G_{\alpha s}$ and $G_{\alpha 1}$ may act through different mechanisms. Boiled $mG_{\alpha 1}$ -GTP γ S (100 pM), GDP-bound $mG_{\alpha 1}$ ($mG_{\alpha 1}$ -GDP, 100 pM), and non-myristoylated $G_{\alpha 1}$ -GTP γ S ($rG_{\alpha 1}$ -GTP γ S; 5–20 nM), had no effect on $G_{\beta 1,2}$ activation of GIRK1 or GIRK1/CIR channels (Fig. 2g). Lack of inhibition by 100 pM $mG_{\alpha 1}$ -GDP, and the fact that $mG_{\alpha 1}$ -GTP γ S suppressed GIRK1 activation despite the great molar excess of $G_{\beta 1,2}$, rule out the possibility of a stoichiometric sequestration of $G_{\beta 1,2}$ by $mG_{\alpha 1}$ -GTP γ S and suggest either an allosteric or a catalytic mechanism for inhibition. Myristoylation is essential for insertion into the membrane of several G_{α} s²² and for $G_{\alpha 1}$ interaction with adenylyl cyclase²³. The efficiency of $mG_{\alpha 1}$ -GTP γ S in cytosol-free membrane patches and the lack of effect of $rG_{\alpha 1}$ -GTP γ S suggest that $mG_{\alpha 1}$ -GTP γ S, like $G_{\beta \gamma}$, acts through interactions involving membrane-associated proteins.

Independent evidence for negative regulation of GIRK1 by $G_{\alpha 1}$ was obtained by injection of antisense oligodeoxynucleotides (ODNs) to reduce the content of G_{α} proteins in oocytes expressing GIRK1 and muscarinic m2 receptor. The whole-cell acetylcholine (ACh)-evoked GIRK1 current, I_{ACh} (Fig. 4a), was increased by phosphorothioate ODNs against *Xenopus* $G_{\alpha 1}$, but not against *Xenopus* $G_{\alpha 3}$, $G_{\alpha 2}$, $G_{\alpha s}$ or $G_{\alpha q}$ (Fig. 4b, d). In addition, the decay of I_{ACh} in the continuous presence of the agonist was strongly suppressed by ODN to $G_{\alpha 1}$, but not to $G_{\alpha 3}$ or $G_{\alpha 2}$ (Fig. 4c), indicating a possible role for $G_{\alpha 1}$ in the desensitization process. ODNs did not alter appreciably the basal activity of GIRK, I_{hK} (Fig. 4e), implying that free, GTP-bound $G_{\alpha 1}$ appears only after receptor activation.

Selective antagonism between $G_{\alpha 1}$ ($G_{\alpha s}$) and $G_{\beta \gamma}$ may participate in determining the specificity of G protein/GIRK interactions, possibly in addition to factors such as compartmentalization and selective G protein/effector association^{7,14,24}. Furthermore, the specific and potent membrane-delimited inhibition of the $G_{\beta \gamma}$ -activated GIRK channel by activated $G_{\alpha 1}$ and $G_{\alpha s}$ described here may serve as a mechanism, previously undetected, for modulation of neuronal excitability. Inhibitory action of activated $G_{\alpha 1}$ and $G_{\alpha s}$ subunits may explain why $G_{\beta \gamma}$ activates GIRK better than GTP γ S in atrial cells⁵ and even more so in oocytes (data not shown). Atrial cells express little $G_{\alpha 1}$ ²⁵, and the role of antagonism between $G_{\alpha 1}$ and $G_{\beta \gamma}$ is uncertain. However, they do coexpress $G_{\alpha s}$ and GIRK, suggesting the possibility that ACh and noradrenaline (through β -adrenergic receptors) counteract each other in atrium not only by the well known opposing actions on the voltage-dependent Ca^{2+} channel²⁶, but also by antagonistic modulation of GIRK. □

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CORRESPONDENCE and requests for materials should be addressed to N. Dascal (e-mail: dascaln@post.tau.ac.il). The ODN sequences with Genbank accession number references were: $G_{\alpha 1}$ (nt 918–891, X56089); $G_{\alpha 3}$ (nt 366–335, X56090); $G_{\alpha 2}$ (918–891, X14636); $G_{\alpha s}$ (931–901, L05540); $G_{\alpha q}$ (119–90, X56091).

Essential role of Stat6 in IL-4 signalling

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INTERLEUKIN-4 (IL-4) is a pleiotropic lymphokine which plays an important role in the immune system¹. IL-4 activates two distinct signalling pathways through tyrosine phosphorylation of Stat6, a signal transducer and activator of transcription, and of a 170K protein called 4PS^{2–7}. To investigate the functional role of Stat6 in IL-4 signalling, we generated mice deficient in Stat6 by gene targeting. We report here that in the mutant mice, expression of CD23 and major histocompatibility complex (MHC) class II in resting B cells was not enhanced in response to IL-4. IL-4-induced B-cell proliferation costimulated by anti-IgM antibody was abolished. The T-cell proliferative response was also notably reduced. Furthermore, production of Th2 cytokines from T cells as well as IgE and IgG1 responses after nematode infection were profoundly reduced. These findings agreed with those obtained in IL-4-deficient mice^{8,9} or using antibodies to IL-4¹⁰ and the IL-4 receptor¹¹. We conclude that Stat6 plays a central role in exerting IL-4-mediated biological responses.

The Stat6 gene disruption was created by the insertion of a

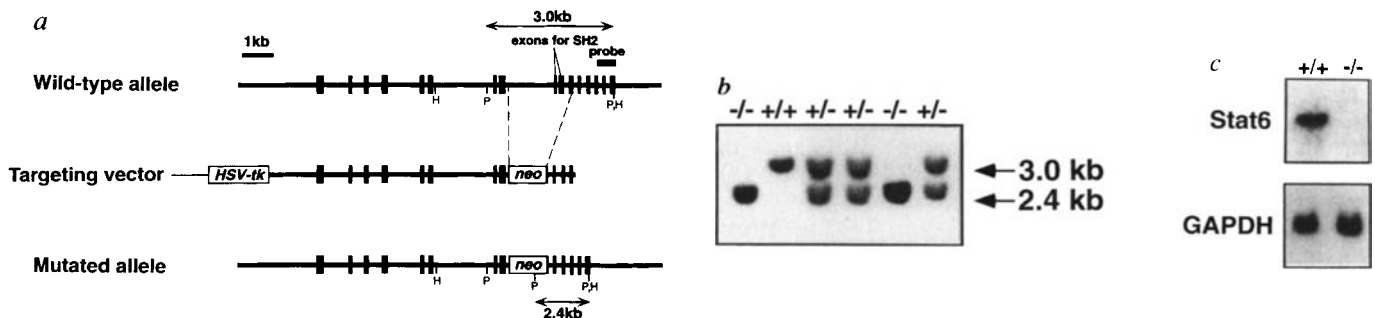


FIG. 1 Disruption of the Stat6 gene. *a*, The structure of the targeting vector and the mutated Stat6 gene. Restriction sites: H, *Hind*III; P, *Pst*I. *b*, Southern blot analysis of the offsprings from intercrosses of Stat6^{-/-} mice. *c*, Northern blot analysis of splenocytes from wild-type and Stat6^{-/-} mice. METHODS. *a*, A murine Stat6 genomic clone was isolated from a strain 129/Sv genomic library and the exon-intron organization was characterized by restriction endonuclease and DNA sequence analysis. The targeting vector was constructed by replacement of a 2.2-kb fragment that contains three exons encoding the SH2 domain of Stat6 with the *neo* resistance gene

(pMC1-*neo*, Stratagene). The herpes-simplex virus thymidine kinase (HSV-TK) gene was inserted into the 5' end of the upstream 7.0-kb fragment. The targeting vector was linearized with *Sa*I and electroporated into E14-1 ES cells. G418- and gancyclovir-resistant clones were screened for homologous recombination by PCR and confirmed by Southern blot hybridization. Generation of chimaeras and mutant mice was essentially as described²⁸. *b*, *Pst*I-digested genomic DNA was hybridized with the probe as shown in *a*. *c*, Total RNA (15 µg) prepared from splenocytes was electrophoresed, transferred to nylon membrane, and hybridized with Stat6 cDNA.

neomycin-resistance (*neo*^r) cassette into three exons of *Stat6* that encode the SH2 domain critical for Stat activation (Fig. 1). Two embryonic stem (ES) cell clones were selected for microinjection into blastocysts. These clones gave germline transmission and were interbred to generate homologous gene targeted mice (Stat6^{-/-}). These mice were viable and showed no overt phenotypic abnormalities. No significant differences in cell numbers or composition of T- and B-lymphocyte subsets were detected between wild-type and Stat6^{-/-} mice (data not shown).

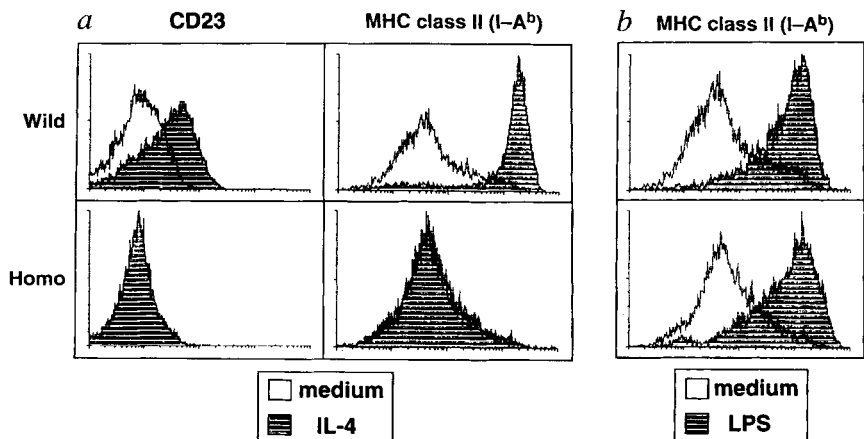
We first examined enhancement of CD23^{15,16} and MHC class II expression^{15,16} on B cells in response to IL-4 (Fig. 2*a*). In wild-type mice, expression of CD23 and MHC class II was increased with stimulation of IL-4 for 48 h. In contrast, B cells from Stat6^{-/-} mice showed no increase of CD23 and MHC class II expression. The expression level of IL-4 receptor on B cells was about the same for wild-type and Stat6^{-/-} mice, which ruled out the possibility that no increase of CD23 and MHC class II expression on Stat6^{-/-} B cells resulted from the downregulation of IL-4 receptor (data not shown). However, the increase of MHC class II expression by lipopolysaccharide (LPS) stimulation was comparable in wild-type and Stat6^{-/-} mice, which shows that impaired augmentation of MHC class II expression in Stat6^{-/-} mice is specific to IL-4 stimulation (Fig. 2*b*). There was also no increase of MHC class II expression in splenic B cells from *Nippostrongylus brasiliensis*-infected Stat6^{-/-} mice (data not shown).

IL-4 acts as a costimulant of B-cell proliferation together with anti-IgM antibodies^{17,18}. IL-4 is also a potent costimulant for normal resting T cells¹⁹. We next analysed the response of IL-4-pretreated small splenic B cells to secondary stimuli (Fig. 3*a*). Small resting B cells preincubated with IL-4 for 24 h were cultured for an additional 48 h in the presence of anti-IgM antibody plus IL-4. Small resting B cells from wild-type mice showed the enhanced proliferation in response to anti-IgM antibody plus IL-4 after preincubation with IL-4. In contrast, no proliferative response was observed with resting B cells from Stat6^{-/-} mice although Stat6^{-/-} B cells proliferated normally in response to LPS. In addition, the proliferation of thymocytes in response to IL-4 plus 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was significantly reduced in Stat6^{-/-} mice, although both thymocytes from wild-type and Stat6^{-/-} mice proliferated equally in response to other stimuli (Fig. 3*b*). These results indicate that Stat6 is involved in proliferation of both B and T cells in the context of a particular activation pathway.

Mature CD4⁺ helper T lymphocytes are categorized into two major functional phenotypes, Th1 and Th2. Th1 cells produce IL-2, interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β), whereas Th2 cells produce IL-4, IL-5 and IL-10²⁰. IL-4 promotes the development of Th2 cells, as clearly demonstrated by the absence of *in vivo* Th2-type immune response development in IL-4^{-/-} mice⁹. We analysed the Th2 cytokine production of T cells

FIG. 2 CD23 and MHC class II expression on small resting B cells. *a*, Small resting B cells were cultured in medium alone or IL-4 (30 U ml⁻¹, Genzyme) for 48 h, and analysed for CD23 and MHC class II expression by flow cytometry. *b*, Small resting B cells were cultured in LPS (5 µg ml⁻¹) for 48 h. Cells were analysed for MHC class II expression.

METHODS. *a*, Spleen cells from 6-week-old mice were treated with anti-Thy1.2 antibody and complement. Small resting B cells were obtained by Percoll gradient centrifugation procedure. Purity of B cells determined by flow cytometry (FACScan, Becton Dickinson) using biotinylated anti-IgM antibody and avidin-FITC. B-cell purity was >95%. After 48 h of culture, cells were stained with FITC-anti-CD23 antibody and biotinylated anti-I-A^b antibody followed by avidin-FITC. *b*, Small resting B cells prepared as described for *a* were cultured for 48 h and stained with biotinylated anti-I-A^b antibody followed by avidin-FITC.



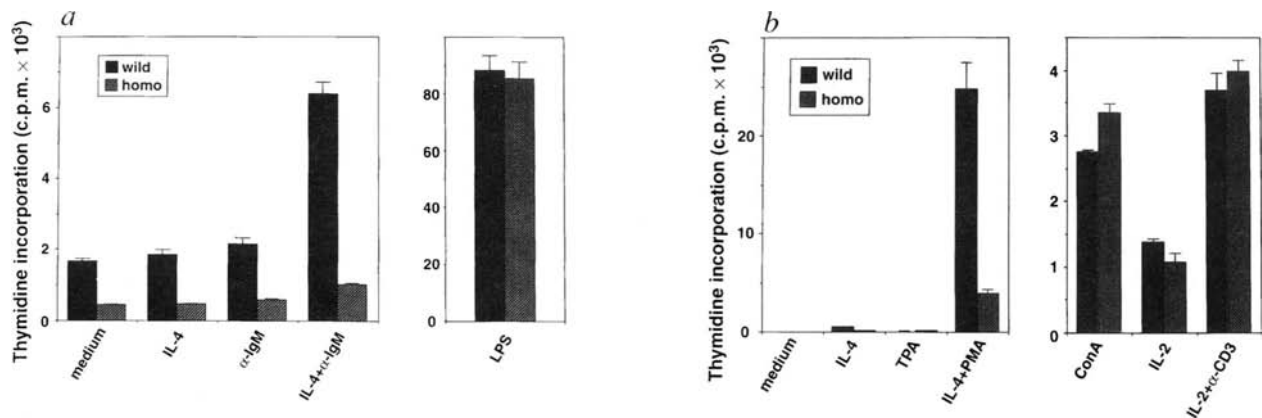


FIG. 3 Proliferative responses of B and T cells to IL-4. *a*, Small resting B cells pretreated with IL-4 were cultured for 48 h in the presence of IL-4 and/or anti-IgM antibody or LPS. Thymidine incorporation was measured. This is the representative data of four repeated experiments. *b*, Thymocytes were cultured for 48 h with the indicated mitogen. Thymidine incorporation was measured. TPA, 12-O-tetradecanoylphorbol-13-acetate; ConA, concanavalin A.

METHODS. *a*, Small resting B cells were prepared as described in Fig. 2. B cells were incubated in 30 U ml⁻¹ IL-4 for 24 h and washed. B cells

(5 × 10⁴) were seeded on 96-well plates and incubated for 48 h in the presence of 30 U ml⁻¹ IL-4, 50 µg ml⁻¹ anti-IgM antibody (Zymed) or 5 µg ml⁻¹ LPS. Cells were pulsed with [³H]thymidine for the last 6 h and incorporation of [³H]thymidine was measured. *b*, Thymocytes (1 × 10⁵) were incubated for 48 h in the presence of 1,000 U ml⁻¹ IL-4, 10 ng ml⁻¹ TPA, 2 ng ml⁻¹ ConA, 10 ng ml⁻¹ IL-2 or 20 µg ml⁻¹ anti-CD3. Cells were pulsed with [³H]thymidine for the last 8 h and incorporation of [³H]thymidine was measured.

from mice infected with *N. brasiliensis* that generates a selective activation of Th2-type immune response²¹ (Fig. 4a). Splenic T cells from *N. brasiliensis*-infected wild-type and *Stat6*^{-/-} mice were stimulated for 24 h with anti-CD3 antibody and supernatants were analysed for cytokine production. In supernatant from T cells obtained from *N. brasiliensis*-infected wild-type mice, the levels of Th2 cytokines such as IL-4, IL-5 and IL-10 were 6- to 30-fold higher than those in pre-immune supernatant. In contrast, Th2 cytokine levels in supernatant from T cells from *N. brasiliensis*-infected *Stat6*^{-/-} mice were not increased. However, IFN-γ production in *N. brasiliensis*-infected *Stat6*^{-/-} mice was not decreased compared with *N. brasiliensis*-infected wild-type mice, indicating the absence of a Th2 response in these mutant mice.

IL-4 is required for immunoglobulin class switching of B cells to IgE and IgG1 producers^{8-11,22}. We measured serum immunoglobulin levels before and at 14 days after *N. brasiliensis* infection (Fig. 4b). In non-infected *Stat6*^{-/-} mice, IgG1 levels were 16-fold lower than those in wild-type mice, and IgE was undetectable. Although IgG1 response was observed in *N. brasiliensis*-infected *Stat6*^{-/-} mice, IgG1 levels were 10-fold lower than those in *N. brasiliensis*-infected wild-type mice. IgE remained undetectable even after *N. brasiliensis* infection in *Stat6*^{-/-} mice. The levels of IgG2, IgG3 and IgA in *N. brasiliensis*-infected *Stat6*^{-/-} mice were slightly increased compared with wild-type control. Similar defects in IgE and IgG1 responses were also observed upon immunization with 2,4-dinitrophenyl-ovalbumin (DNP-OA)

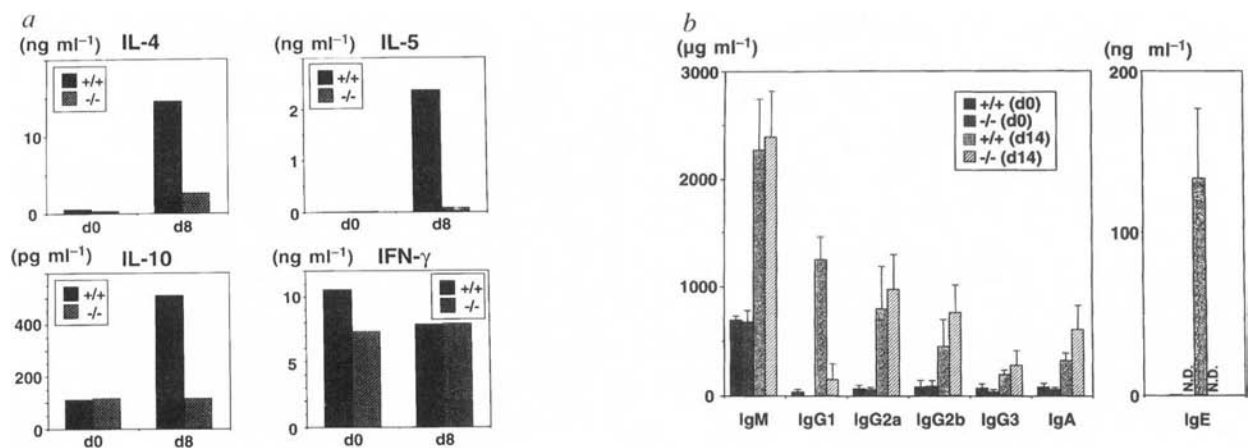


FIG. 4 Cytokine production from splenic T cells and serum immunoglobulin levels in *N. brasiliensis* infection. *a*, Cytokine secretion from splenic T cells. Splenic T cells of pre-(d0) and post-(d8) *N. brasiliensis*-infected mice were stimulated for 24 h with anti-CD3 antibody. Supernatants were analysed for production of IL-4, IL-5, IL-10 and IFN-γ by ELISA. Representative data of two repeated experiments. *b*, Serum levels of immunoglobulins were measured before (d0) and at 14 days after *N. brasiliensis* infection (d14). N.D., not detected.

METHODS. Eight-week-old mice were subcutaneously injected with 750 third-stage larvae of *N. brasiliensis*. Eight days after infection splenic T cells were enriched by nylon wool column passage from infected and non-

infected mice. There was no significant difference in the number of splenic T cells between wild-type and *Stat6*^{-/-} mice. Cell purity was determined by flow cytometry (FACScan, Becton Dickinson) using FITC-labelled anti-CD3 monoclonal antibody 2C11. T-cell purity was > 90%. CD3⁺ cells (1 × 10⁶ cells) were cultured on anti-CD3 (20 µg ml⁻¹ monoclonal antibody 2C11 in Hanks' balanced salt solution)-coated dishes for 24 h. After 24 h of culture, supernatants were collected. Cytokine production was analysed with ELISA kit (Genzyme and Endgen). For serum immunoglobulin levels, sera were obtained from *N. brasiliensis*-infected mice before and at 14 days after infection (six mice per group), and analysed by ELISA.

(data not shown). Taken together the data presented in this study completely agreed with those seen in *IL-4*^{-/-} mice or using antibodies to IL-4 and the IL-4 receptor⁸⁻¹¹.

IL-4 exerts various biological effects by regulating proliferation and differentiation of a wide variety of lymphoid and myeloid cells¹. Activation of the IL-4 receptor involves interaction of the ligand with both the IL-4 receptor α -subunit (IL-4R α) and the common γ -chain which is also a component of the IL-2 and IL-7 receptors^{3,24}. Two distinct tyrosine phosphorylated proteins, Stat6 and IRS-related 4PS play a pivotal role in the signalling pathway mediated by IL-4²⁻⁷. Phosphorylated 4PS interacts with the regulatory subunit of PI-3K(p85), potentially GRB-2, as well as other molecules containing the SH2-domain^{23,25}. The haematopoietic

cell line 32D, deficient in 4PS fails to proliferate in response to IL-4, providing evidence that 4PS is indispensable for IL-4-stimulated mitogenesis²⁶. Furthermore, several findings indicate that the Jak-Stat signalling pathway contributes to specific gene expression but not mitogenesis^{6,27}. However, our present data with *Stat6*^{-/-} mice show that besides specific gene expression, Stat activation is actually involved in proliferation, at least in the IL-4R system, demonstrating that the signalling pathway through Stat6 activation is the predominant pathway that leads to the biological responses induced by IL-4. As Stat6 has been shown to be specifically phosphorylated by IL-4, agents that block the function of Stat6 may be therapeutically useful for IL-4-mediated disorders such as allergy and atopy. □

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Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene

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SIGNAL transducers and activators of transcription (Stats) are activated by tyrosine phosphorylation in response to cytokines, and are thought to mediate many of their functional responses¹⁻⁴. Stat6 is activated in response to interleukin (IL)-4 (refs 5,6) and may contribute to various functions including mitogenesis, T-helper cell differentiation and immunoglobulin isotype switching⁷. To evaluate the role of Stat6, we generated Stat6-null mice (*Stat6*^{-/-}) by gene disruption in embryonic stem cells. The mice were viable, indicating the lack of a non-redundant function in normal development. Although naive lymphoid cell development was normal, *Stat6*^{-/-} mice were deficient in IL-4-mediated functions including Th2 helper T-cell differentiation, expression of cell surface markers, and immunoglobulin class switching to IgE. In contrast, IL-4-mediated proliferation was only partly affected.

We disrupted the murine *Stat6* gene by replacing the first coding exon with a *neo* resistance gene cassette (Fig. 1a). Appropriately

targeted embryonic stem (ES) cell clones were used to obtain chimeric mice that transmitted the disrupted locus through the germ line. Breeding of *Stat6*^{+/-} mice resulted in *Stat6*^{-/-} progeny, which displayed no obvious abnormalities, at the expected mendelian frequency (Fig. 1b). No differences were observed in lymphoid or myeloid cell numbers or expression of markers including B220, IgM, CD3, CD4, CD8, CD14, GR-1 or CD34 (data not shown).

Immunoprecipitation and western blotting with a Stat6 carboxy-terminal antiserum detected the expected protein of relative molecular mass 100,000 (*M*_r 100K) in wild-type mice but not in *Stat6*^{-/-} mice (Fig. 1c). But *Stat6*^{-/-} mice did have a minor, smaller protein, possibly derived from a residual transcript. IL-4 induced Stat6 tyrosine phosphorylation and DNA binding activity in spleen cells from wild-type mice but not from *Stat6*^{-/-} mice (Fig. 1c, d, respectively).

We first examined lymphoid proliferation, but detected no differences between *Stat6*^{-/-} and wild-type mice in the response of spleen, bone marrow or thymus cells to concanavalin A or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (data not shown). IL-4 significantly stimulated proliferation of anti-IgM-treated splenic B cells from *Stat6*^{-/-} mice, although at a level one-third that of wild-type mice (Fig. 2a, left; *P* = 0.0124). IL-4, with submitogenic TPA concentrations, also stimulated B cells from *Stat6*^{-/-} mice, although the response was lower than that of wild-type mice, and was not statistically significant. No statistically significant differences were observed between wild-type and *Stat6*^{-/-} mice in the responses of CD4⁺-enriched T cells to IL-4 and submitogenic concentrations of TPA (Fig. 2a, right).

The effects on major histocompatibility complex (MHC) class II, Thy-1 or CD23 (Fc ϵ RII) expression on B cells⁷ are shown in Fig. 2b. Although MHC class II expression on untreated cells was similar for wild-type and *Stat6*^{-/-} mice, IL-4-induced upregulation of MHC class II antigens was greatly reduced with *Stat6*^{-/-} B cells (Fig. 2b, top). Untreated *Stat6*^{-/-} B cells consistently expressed lower levels of CD23 than B cells from wild-type mice (data not shown). When B cells from wild-type mice were cultured with lipopolysaccharide (LPS) and IL-4, the frequency of cells expressing high levels of CD23 was increased (Fig. 2b, middle) but not with B

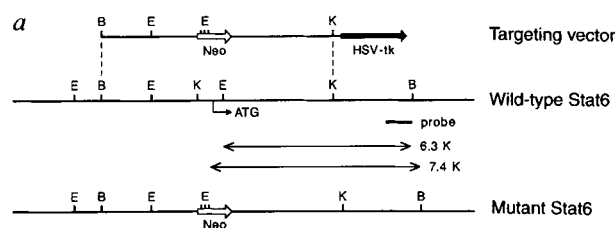
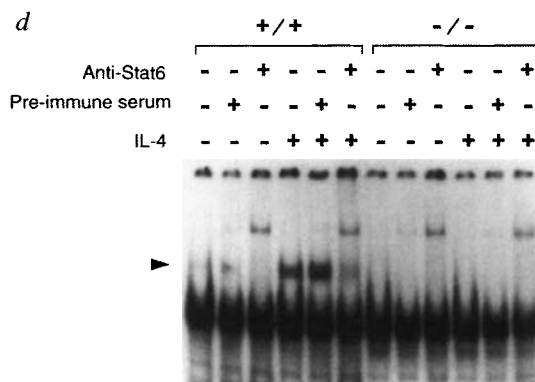
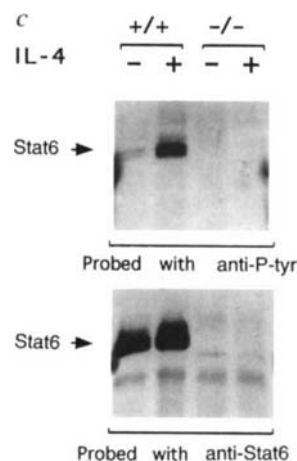
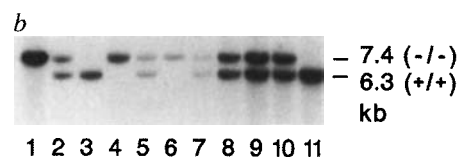


FIG. 1 *Stat6* gene disruption. *a*, The targeting vector (top), a *Stat6* restriction site map (middle), and the homologous recombination (bottom) are shown. A replacement targeting vector containing 3.1 kb and 3.6 kb of 5' and 3' homology was constructed for positive (Neo) and negative selection (thymidine kinase). Restriction sites are *Bam*HI (B), *Eco*RI (E) and *Kpn*I (K). *b*, Southern blot analysis of F2 DNA. *Bam*HI-*Eco*RI-digested DNAs were hybridized with the 0.9-kb *Xho*I-*Bam*HI-indicated probe. The endogenous and targeted alleles are 6.3 kb and 7.4 kb, respectively. *c*, Immunoblot analysis of Stat6. Spleen cells from wild-type or *Stat6*^{-/-} mice were stimulated with or without IL-4 and lysed. Stat6 was immunoprecipitated with a C-terminal antiserum and analysed by western blotting with a monoclonal antibody against phosphotyrosine (top) or Stat6 antiserum (bottom). *d*, Stat6 DNA binding activity. Spleen cells from wild-type or *Stat6*^{-/-} mice were incubated in LPS for 24 h and stimulated with or without IL-4 for 30 min and lysed. Extracts were analysed by gel-shift analysis and supershifting with Stat6 antiserum. The position of the IL-4-induced DNA binding complex is indicated.

METHODS. *a*, Genomic clones were from a 129-derived CCE ES cell library. The neomycin-resistance gene cassette (Neo) pMC1 (Stratagene) replaced a 0.8-kb *Kpn*I-*Eco*RI fragment containing the entire first *Stat6* coding exon. A herpes simplex thymidine kinase cassette (HSV-tk) was used for negative selection. *b*, Southern blots were as described²⁸. *c*, Spleen cells were cultured with 25 μ g ml⁻¹ of LPS for 24 h and were untreated or were stimulated 30 min with 50 U ml⁻¹ IL-4. Extracts were assayed as described⁶. *d*, Spleen cells were obtained as in *c*, and extracts obtained for DNA binding as described⁶. The probe used (5'-GATCAAGACCTTTTCCCAAGAAATCTATC-3') contains the GAS-like sequence found in the promoter of the human *C α* gene.



cells from *Stat6*^{-/-} mice. IL-4 alone also induced CD23 on B cells from wild-type mice, but not on cells from *Stat6*^{-/-} mice (data not shown). Finally, the IL-4-induced expression of Thy-1 on LPS-treated B cells from wild-type mice⁸ was not seen with *Stat6*^{-/-} cells (Fig. 2*b*, bottom).

IL-4 facilitates the differentiation of naive, CD4⁺ T cells into Th2 cells that secrete IL-4, IL-5, IL-10 and IL-13 (refs 9,10) after antigen stimulation. In the absence of IL-4, Th1 cells develop that characteristically produce interferon (IFN)- γ , and this is enhanced by IL-12. We therefore used an *in vitro* assay⁹ to assess the consequences of *Stat6* deletion on Th differentiation. Enriched splenic CD4⁺ T cells were cultured for five days (as indicated in Fig. 3 legend) to promote Th1 or Th2 differentiation. The cells were restimulated and cytokine production measured. In cultures supporting Th1 differentiation, restimulation resulted in production of IFN- γ (Fig. 3), and there were no differences between wild-type and *Stat6*^{-/-} mice. Restimulation of cells from wild-type mice under conditions that promote Th2 differentiation resulted in the production of IL-4, IL-5 and IL-10, and very low levels of production of IFN- γ (Fig. 3). In contrast, IL-4,

IL-5 and IL-10 production was dramatically reduced in CD4⁺ T cells from *Stat6*^{-/-} mice. Moreover, the decreased IFN- γ production seen in wild-type mice was not seen in the *Stat6*^{-/-} mice, suggesting a lack of IL-4-mediated suppression of Th1 type differentiation.

IL-4 influences B-cell immunoglobulin class switching to IgG1 and IgE^{7,11,12}. To assess this, we examined the response of anti-IgD-treated mice in which class switching is induced¹³. Unexpectedly, there were no detectable differences between wild-type and *Stat6*^{-/-} mice in the serum levels of IgG1, IgG2A, IgG2B, IgG3, IgA or IgM before IgD treatment (Fig. 4*a*). In untreated *Stat6*^{-/-} mice there was no detectable IgE, but low levels were detected in untreated wild-type mice. Treatment of wild-type mice with anti-IgD induced a dramatic increase in serum IgG1 and IgE. Treatment of *Stat6*^{-/-} mice resulted in a comparable increase in IgG1, but only very low levels of IgE were detected. In addition, there was an increase in serum IgG2a, IgG2b and IgG3 in anti-IgD-treated *Stat6*^{-/-} mice relative to wild-type mice. We also examined the numbers of lymph-node B cells producing various immunoglobulin isotypes (Fig. 4*b*). Consistent with the serum antibody

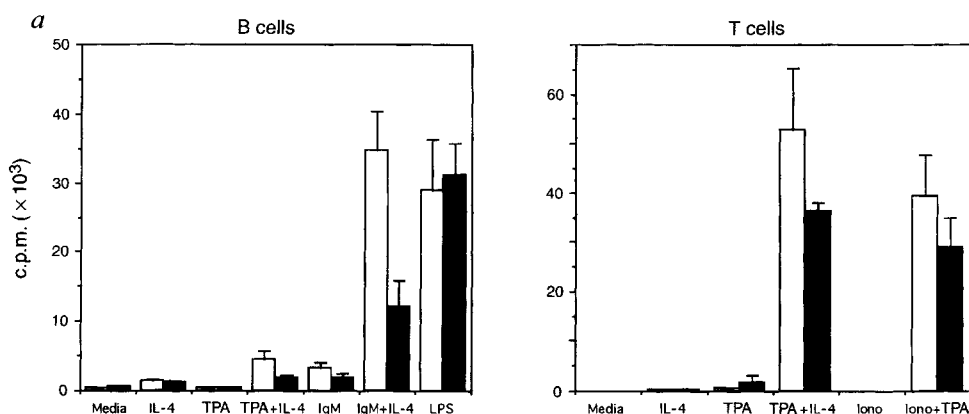


FIG. 2 Lymphoid functions in *Stat6*^{-/-} mice. **a**, Lymphocyte proliferation. Splenic B cells from wild-type mice (white bars) or *Stat6*^{-/-} mice (black bars) were cultured in medium, IL-4 (250 U ml⁻¹), anti-IgM (5 µg ml⁻¹), submitogenic concentrations of TPA (0.5 ng ml⁻¹), LPS (20 µg ml⁻¹), IL-4 with TPA or IL-4 with anti-IgM (left). Splenic CD4⁺-enriched T cells from wild-type mice (white bars) or *Stat6*^{-/-} mice (black bars) were stimulated with IL-4 (1,000 U ml⁻¹), submitogenic concentrations of TPA (2 ng ml⁻¹), ionomycin (100 ng ml⁻¹) and TPA or IL-4 with TPA (right). After 2 days, DNA synthesis was measured by [³H] thymidine incorporation for 8 h. Values are the mean ± s.e.m. c.p.m. incorporated from 6–8 mice. Proliferation was measured with 5–6 replicates with less than 15% variation. A Student's *t*-test was used for statistical evaluations. **b**, Flow-cytometric analysis of IL-4-stimulated B cells. Splenic B cells were cultured with medium (dotted line) or IL-4 (solid line) (top); LPS (dotted line) or IL-4 plus LPS (solid line) (middle and bottom) for 2 days. Expression of MHC class II (top), CD23 (middle) or Thy-1 (bottom) was analysed.

METHODS. **a**, Splenic lymphocytes were treated with a monoclonal antibody against Thy-1 for 30 min on ice followed by incubation with guinea-pig and rabbit complement (Cedarlane) for 60 min at 37 °C and small, dense B cells were collected by Percoll gradient centrifugation. CD4⁺ T cells were prepared by removal of CD8⁺ T cells and Ig⁺ B cells using immunomagnetic beads. B cells (5 × 10⁴ per well) or T cells (2.5 × 10⁴ per well) were stimulated and assayed as indicated. **b**, B cells were prepared as in **a**. After stimulation with or without 50 U ml⁻¹ IL-4 for 24 h, MHC class II expression was assessed by fluorescence-activated cell sorting. Alternatively, B cells were cultured in the presence of 20 µg ml⁻¹ LPS with or without 100 U ml⁻¹ of IL-4 for 48 h for the detection of CD23 and Thy 1. Cells (1 × 10⁶) were stained with anti-CD45R/B220 in the presence of anti-MHC class II, anti-CD23 or anti-Thy-1. Gates were set on CD45R/B220 positive cells. Antibodies were purchased from Pharmingen.

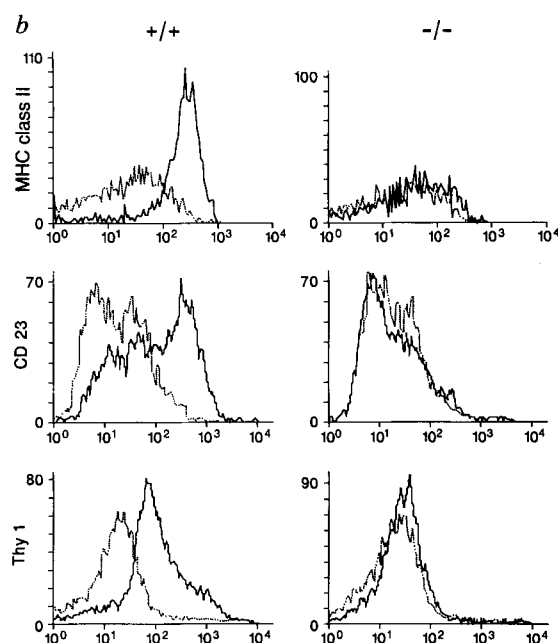
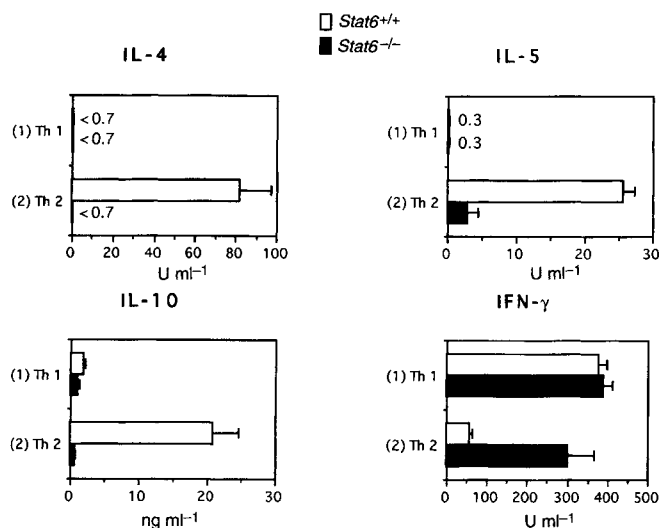


FIG. 3 *In vitro* T helper cell differentiation. CD4⁺ T cells were stimulated under the conditions indicated for 5 days, washed and restimulated with anti-CD3, anti-CD28 and IL-2 for 24 h. The cytokine levels of 24-h supernatants were assayed by enzyme-linked immunosorbent assay (ELISA) and standardized to commercial cytokines (IL-10 from R&D; IL-4, IL-5 and IFN-γ from Pharmingen). Values are given as ng ml⁻¹ for IL-10, and U ml⁻¹ for IL-4, IL-5 and IFN-γ. The detection level of IL-4 is 0.7 U ml⁻¹ and of IL-5 is 0.1 U ml⁻¹.

METHODS. Spleen cells were cultured on plastic plates for 1 h at 37 °C to remove macrophages. Non-adherent cells were reacted with mouse or rat monoclonal antibodies to CD8 (19.178) and class II antigen at 4 °C for 1 h. The cells were then cultured in plates precoated with a goat anti-rat immunoglobulin and rabbit anti-mouse immunoglobulin at 4 °C for 1 h. Purified CD4⁺ cells were cultured on 20 µg ml⁻¹ of anti-CD3ε (145.2C11) plus anti-CD28 (10 µg ml⁻¹, 37.51, Pharmingen) coated dishes in the presence of 50 U ml⁻¹ of IL-2 (Genzyme) and either anti-IL-4 (40 µg ml⁻¹, 11B11) and IL-12 (1 ng ml⁻¹, R&D) to promote Th1 differentiation or IL-4 (50 ng ml⁻¹) to promote Th2 differentiation. After 5 days the cells were washed and restimulated for 24 h as before in 1 × 10⁶ cells in 1 ml of media containing 50 U ml⁻¹ of IL-2, anti-CD3 and anti-CD28.



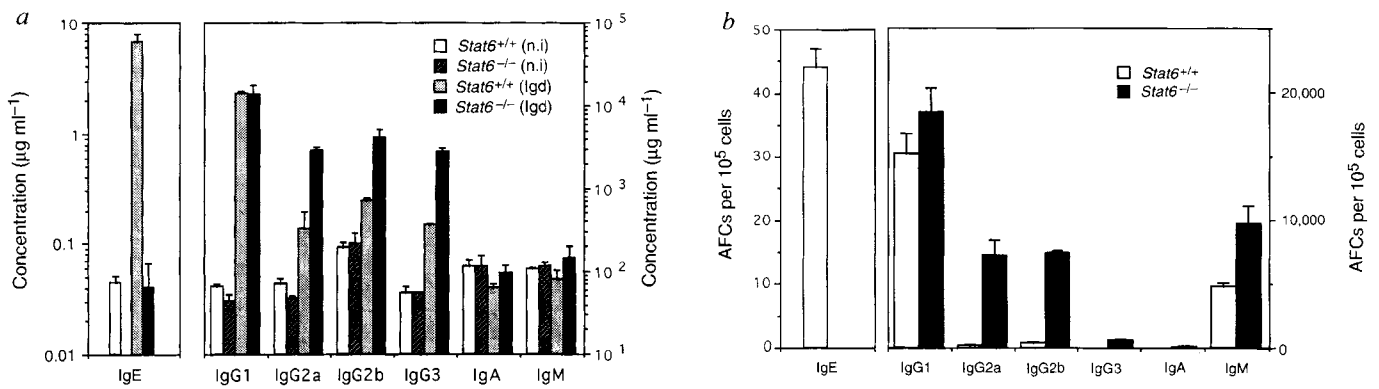


FIG. 4 Serum levels of immunoglobulins and frequency of immunoglobulin-secreting cells in anti-IgD-treated mice. *a*, Serum concentrations of immunoglobulin isotypes in wild-type and *Stat6*^{-/-} mice before (3 mice) and after (2 mice) anti-IgD treatment. Error bars indicate the assay variation. *b*, An enzyme-linked immunospot (ELISPOT) assay was done to enumerate antibody-secreting cells in the cervical lymph nodes from anti-IgD-treated mice. Values shown are immunoglobulin antibody-forming cells (AFCs) per 1 × 10⁵ cells, and are derived from two individually assayed mice. Sample error is indicated.

METHODS. Mice were subcutaneously injected with 100 μl of goat anti-mouse IgD antiserum, and serum was obtained before injection or at 8 days. *a*, For ELISA assays, plastic plates were coated with goat anti-mouse immunoglobulin (Southern Biotechnology) for the detection of IgG1, IgG2a, IgG2b, IgG3, IgA and IgM. IgE was captured with a rat anti-mouse IgE (clone R35-72, Pharmingen). Serially diluted serum samples were added and

bound immunoglobulin was detected with alkaline phosphatase-conjugated isotype-specific goat anti-mouse antibody or rat anti-mouse IgE monoclonal antibody (clone 23G3, Southern Biotechnology). Concentrations were calculated using purified immunoglobulins as standards. The assay can detect > 0.015 μg ml⁻¹ IgE. *b*, For the ELISPOT assay, goat anti-mouse antibody κ light chain was used as the capture antibody. Cervical lymph node cells (5 × 10⁵, and fivefold dilutions) from mice treated 8 days previously with anti-IgD were added to individual wells and incubated for 4 h at 37 °C. After washing, alkaline phosphatase-conjugated, isotype-specific goat anti-mouse antibody was added. Plaques of cells producing individual isotypes were counted. IgE-producing cells were detected by using a rat anti-mouse IgE monoclonal antibody (clone R35-72, Pharmingen) for capture and rat anti-mouse IgE monoclonal antibody (clone 23G3, Southern Biotechnology) for detection.

levels, anti-IgD-treated *Stat6*^{-/-} mice contained no detectable IgE-producing B cells. Furthermore, although IgG1, IgA and IgM antibody-producing cells were comparable, there was a consistent increase in IgG2a, IgG2b and IgG3 antibody-producing cells. Together these results demonstrate that *Stat6* is essential for IgE class switching, and that it negatively affects switching to IgG2a, IgG2b and IgG3.

The phenotype of *Stat6*^{-/-} mice is striking in several regards. The relative lack of an effect on IL-4 comitogenic activity is consistent with studies with IL-4 receptor mutants demonstrating that, although Jak and IRS-1 activation are critical for mitogenesis, Stat6 activation is not^{6,14,15}. The B cell-specific, partial reduction in comitogenic activity may be related to effects on differentiation. In *Stat6*^{-/-} mice, IL-4 failed to induce upregulation of MHC class II antigens and CD23, which is consistent with studies implicating Stat-like activities in their expression¹⁶⁻¹⁹. In contrast, little is known about the regulation of Thy-1 expression.

Th1 and Th2 cells play unique roles in immune responses^{7,20}, and the role of IL-4 in Th2 differentiation was indicated by IL-4-deficient mice²¹. Our results demonstrate that Stat6 is critical for

the gene regulation involved in helper T-cell differentiation. IL-12, which supports the differentiation of Th1 cells, uniquely activates Stat4 (ref. 22) and, in the initial characterization of *Stat4*^{-/-} mice, Th1 differentiation is specifically affected (W. Thierfelder *et al.*, unpublished data). Thus two members of the Stat family may provide fundamental control of T helper cell differentiation. The inability of *Stat6*^{-/-} mice to make IgE supports the hypothesis that Stat6-driven *Cε* gene transcription is critical for class switching^{17,18,23,24}. It is surprising that we found no effect on IgG1 switching similar to that seen in IL-4^{-/-} mice²¹; this could reflect either an IL-4-independent control of IgG1 switching, or an IL-4-dependent effect unrelated to Stat6.

The lack of other alterations in *Stat6*^{-/-} mice was unexpected because both IL-3 (ref. 6) and IL-13 (ref. 25) activate Stat6, and Stat6 is broadly expressed⁶. The similarity of *Stat6*^{-/-} mice to IL-4^{-/-} mice suggests that Stat6 evolved for IL-4-specific functions, not unlike Stat1 which, based on the phenotype of *Stat1*^{-/-} mice^{26,27}, evolved for IFN-specific functions. Irrespective of this, it will be interesting to assess the susceptibility of *Stat6*^{-/-} mice to various infectious agents and autoimmune diseases to understand further the contribution of Stat6 to acquired immunity. □

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Calcium mobilization via sphingosine kinase in signalling by the FcεRI antigen receptor

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CALCIUM mobilization through antigen receptors, including high-affinity IgE receptors (FcεRI), is thought to be mediated by inositol-1,4,5-trisphosphate production (InsP₃)¹⁻⁴. Here we show that antigen clustering of FcεRI on the rat mast-cell line (RBL-2H3) activates a sphingosine kinase (SK) and produces sphingosine-1-phosphate (S1P), an alternative second messenger for intracellular calcium mobilization. The sphingosine analogue, D,L-threo-dihydrosphingosine (DHS), inhibits the SK enzyme competitively with a dissociation constant, *K_i*, of 5 to 18 μM. This inhibition substantially suppresses the FcεRI-mediated calcium signal, but leaves intact the *syk* tyrosine kinase activation and the small InsP₃ production. The entire InsP₃-dependent pathway activated by a transfected G-protein coupled receptor, used here as a positive control, also remained intact. Thus FcεRI principally utilizes a SK pathway to mobilize calcium.

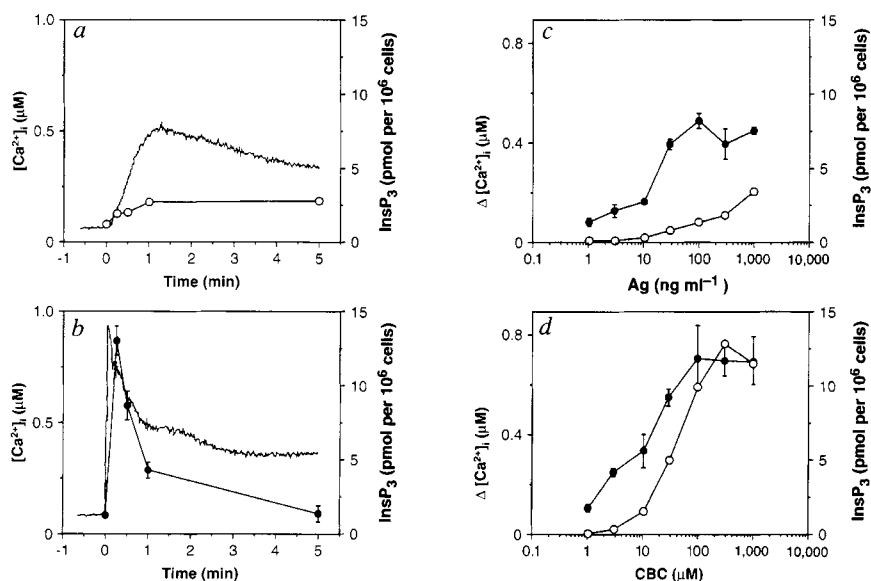
RBL-2H3 cells (RBL-m1) transfected with m1 muscarinic receptor (m1R)^{5,6} were first incubated with anti-dinitrophenyl immunoglobulin E (anti-DNP IgE), before challenge with the multivalent antigen dinitrophenylated human serum albumin (DNP-HSA) or with carbachol (CBC). As expected from previous studies⁶, the InsP₃ signal after CBC challenge was more than ten times greater than after antigen challenge (Fig. 1a, b). In contrast,

the calcium mobilization by both receptors, although of different kinetics, was of the same order of amplitude. We then calibrated the InsP₃ production with cytosolic calcium increase at the time of DNP-HSA or CBC stimulation at which cytosolic calcium was maximum (Fig. 1c, d). There was a good correlation between InsP₃ production and calcium increase in CBC-stimulated cells. However, the InsP₃ increases generated by both receptors resulted in different calcium increases. For example, a maximal calcium increase of about 0.5 μM corresponds to the formation of about 1.5 pmol InsP₃ per 10⁶ cells after antigen stimulation, but almost 4 pmol InsP₃ per 10⁶ cells for G-protein-linked receptor. Thus there is an InsP₃ gap between the two receptors, which suggests that there is an alternative calcium signalling pathway used by FcεRI.

Introduction of sphingosine-1-phosphate (S1P) into DDT₁MF-2 smooth-muscle cell line, as well as Swiss 3T3 fibroblasts, has been shown to induce a calcium response^{7,8}. Sphingosine, the precursor of S1P, has also been shown to induce calcium response in permeabilized RBL-2H3 cells in an InsP₃-independent manner⁹. Because S1P is generated through the activation of SK, we investigated whether activation of SK¹⁰⁻¹³, and the production of S1P, could be induced by FcεRI and be an alternative pathway to calcium signalling. To measure SK activity, RBL-m1 cells were loaded with anti-DNP IgE and stimulated with DNP-HSA; the cells were lysed and the SK activity towards the production of S1P determined. To measure *in vivo* production of S1P, cells loaded with anti-DNP IgE were preincubated with [³²P]orthophosphoric acid and stimulated with DNP-HSA. Labelled lipids were extracted and resolved by two-dimensional thin layer chromatography (TLC). Engagement of FcεRI with antigen resulted in a very rapid stimulation of SK activity, reaching its maximum (around 100% increase of the basal activity) within 15 s of stimulation (Fig. 2a). By comparison with the enzyme activity, the maximal *in vivo* production of S1P was delayed by about 1 min. Both activation of SK and the production of S1P were dependent on antigen dose (Fig. 2b). The activation of SK by carbachol was less than 20% of that for antigen (data not shown).

Study of the *in vitro* or *in vivo* activation of SK indicated that it follows a Michaelis-Menten kinetic. Similar *K_m* values of activation were obtained after *in vitro* (21.4 μM sphingosine) or *in vivo*

FIG. 1 Comparison between FcεRI- and m1R-mediated production of InsP₃ and calcium mobilization. a, b, Time course of calcium signal (trace) induced by 1 μg ml⁻¹ Ag (a) or by 1 mM CBC (b) was compared with that of InsP₃ production (a, open circles; b, filled circles). c, d, Cytosolic free calcium increase (filled circles) above the background and InsP₃ increase above the baseline (open circles) was measured 1.5 min or 15 s after stimulation with various concentrations of Ag (c) or CBC (d), respectively. RBL-m1 cells were maintained in growth media containing 15% fetal bovine serum, 2 mM glutamine and antibiotics. Cells were loaded with anti-DNP IgE overnight and the experiment was performed in a glucose-saline, PIPES buffer (pH 7.2), containing (in mM) 25 PIPES, 110 NaCl, 5 KCl, 5.6 glucose, 0.4 MgCl₂, 0.1% BSA and 1 CaCl₂. For the measurement of InsP₃ generation, cells grown in culture dishes were stimulated at 37 °C with either DNP-HSA (Ag) or carbachol (CBC) for the indicated times, and the reaction was quenched by adding ice-cold 20% trichloroacetic acid. The amount of InsP₃ was determined according to the manufacturer's instructions provided with the InsP₃ assay kit (Dupont/NEN, Boston), except that the aqueous phase of the extract was passed through ultrafiltration units to exclude proteoglycans that interfere with the assay⁶. Values are mean ± s.e.m. For the measurement of the cytosolic free calcium, cells preloaded with anti-DNP IgE were collected and incubated in the complete growth medium for 2 h at 37 °C with gentle rotation. The cells were then loaded with 2 μM Fura-2 (Molecular Probe) for 45 min and measurement of cytosolic calcium was performed in a buffer,



containing (in mM) 135 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 5.6 glucose, 10 HEPES, 2.5 probenecid and 0.1% BSA, in a Deltascan bulk spectrofluorimeter (Photon Technology Inc.) as previously described¹⁷. Traces are representative of more than 3 experiments. Cytosolic free calcium increase was calculated from 3 or more traces. Values are mean ± s.d.

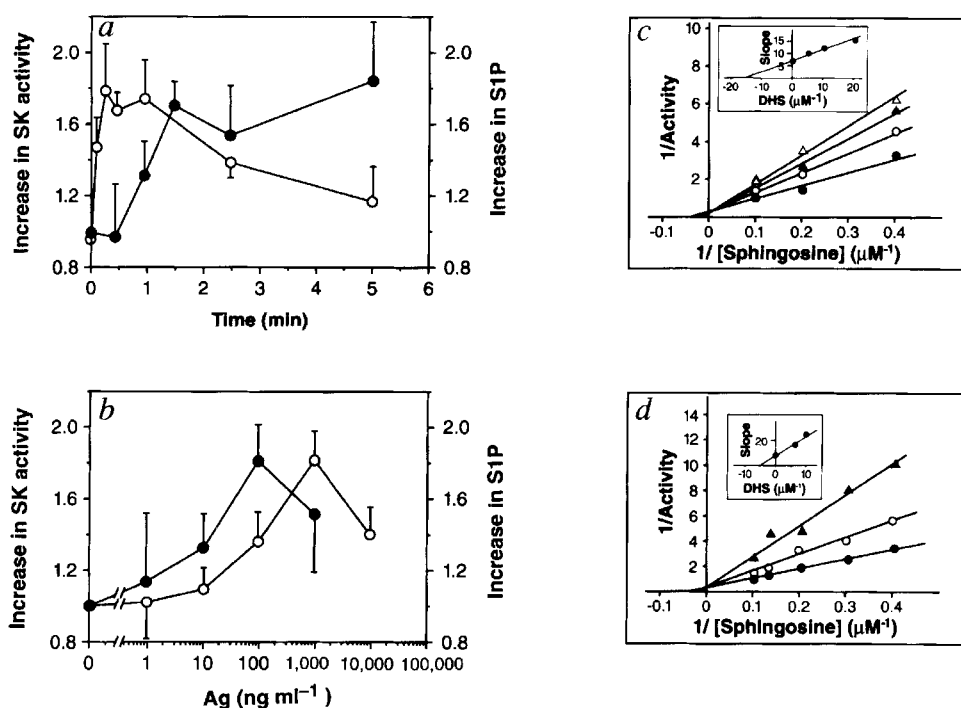


FIG. 2 DNP-HSA (Ag)-induced SK activity and production of S1P are time- and dose-dependent. *a*, Cells were stimulated with either $1 \mu\text{g ml}^{-1}$ Ag for SK assay or 100 ng ml^{-1} for S1P measurement for the indicated times. Concentrations of Ag for time-course study were chosen to give maximal response based on the dose response as shown in *b*. *b*, Cells were stimulated with indicated concentrations of Ag for 15 s for SK assay or 1.5 min for S1P measurement. *c*, For *in vitro* kinetic study of SK activity, cell extract was prepared as mentioned for SK assay and kinase assay was performed with various concentrations of sphingosine (2.5, 5 and $10 \mu\text{M}$) in the absence (filled circles) or presence of $5 \mu\text{M}$ (open circles), $10 \mu\text{M}$ (filled triangles) or $20 \mu\text{M}$ (open triangles) of DHS. S1P generated *in vitro* was measured and calculated as a fraction of the amount obtained with $10 \mu\text{M}$ sphingosine in the absence of DHS. A double reciprocal plot is shown and x-

($30 \mu\text{M}$ sphingosine) determination (Fig. 2*c, d*). The kinetics of inhibition of the SK enzyme by the sphingosine analogue DHS was characteristic of competitive inhibition as shown in other systems^{14,15}. Double reciprocal plots were replotted (DHS concentration versus slope) to determine the K_i values (Fig. 2*c, d*, insets). Same-order values were obtained after *in vitro* ($18 \mu\text{M}$) or *in vivo* ($5 \mu\text{M}$) determinations.

In agreement with previous reports in other cell lines^{7,8}, S1P could by itself induce a calcium response in intact RBL-m1 cells (Fig. 3*a*). This increase in calcium was not inhibited by pretreatment of the cell with EGTA, indicating that this calcium release was from intracellular stores. In addition, DHS did not inhibit the S1P-induced calcium signal, a further indication that DHS acts at the level of the SK enzyme and not at the level of the putative S1P receptors.

To document further the linkage between SK activation and calcium signalling, we investigated the effect of the SK inhibitor DHS on the calcium signalling pathways initiated by both FcεRI and m1R. The calcium signal after antigen stimulation was markedly inhibited, albeit not completely, whereas subsequent addition of carbachol elicited a normal m1R calcium mobilization (Fig. 3*b*). In addition, under the same conditions, the *in vivo* formation of S1P mediated by FcεRI was completely abolished by DHS (Fig. 3*b*, inset). The effect of DHS on InsP_3 generation was then investigated to eliminate the possibility that DHS might inhibit phospholipase C *in vivo* and reduce the generation of InsP_3 . The SK inhibitor had no effect on the small FcεRI-mediated InsP_3 generation or on the large production of InsP_3 after m1R activation (Fig. 3*c*). To rule out a direct effect of DHS on tyrosine

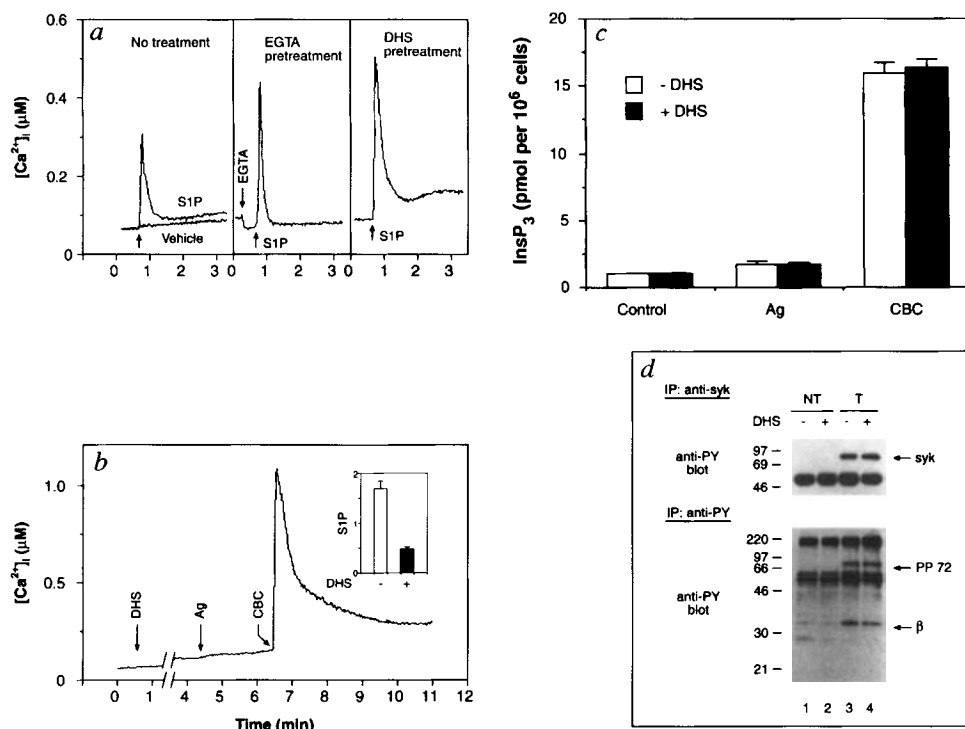
intercept of the line obtained in the absence of DHS gives $-1/K_m$. To determine the dissociation constant, K_i , for DHS, slope was calculated from each line from the double reciprocal plot and slope was plotted versus DHS concentration. The x-intercept gives $-K_i$ (inset). *d*, For *in vivo* kinetic study of SK activity, cells prelabelled with [^{32}P]orthophosphoric acid were stimulated with various concentrations of sphingosine (2.5, 3.33, 5, 7.5 and $10 \mu\text{M}$) in the absence (filled circles) or presence of $5 \mu\text{M}$ (open circles) or $10 \mu\text{M}$ (filled triangles) or DHS, and *in vivo* formation of [^{32}P]S1P was quantified as described above. Data were analysed as in *c*. SK activity was measured as previously described with some modifications¹⁰. After stimulation, cells were lysed by freeze-thawing in 0.1 M phosphate buffer (pH 6.8) containing (in mM) 10 MgCl_2 , 20% glycerol, 1 mercaptoethanol, 1 EDTA, $20 \mu\text{M}$ ZnCl_2 , 1 Na_2VO_4 , 15 NaF, $10 \mu\text{g ml}^{-1}$ leupeptin and aprotinin, 1 PMSF and 0.5 4-deoxy pyridoxine. Cytosolic fractions were prepared by ultracentrifugation at $105,000g$ for 90 min. Sphingosine kinase activity was measured by incubating the supernatant with $5 \mu\text{M}$ sphingosine-BSA complex and [^{32}P]ATP (1 mM , 2 mCi ml^{-1}) for 15 min at 30°C . Labelled lipids were extracted and separated by TLC along with S1P standard. Radioactivity of the spot corresponding to S1P was quantified using Ambis Radioanalytical and Optical Imaging System. Values are expressed as increase relative to the radioactivity in unstimulated cultures and mean $\pm$ s.d. of 3 or more experiments. *In vivo* measurement of S1P production was performed as previously described with modifications¹⁴. Cells preloaded with DNP-IgE were preincubated with [^{32}P]orthophosphoric acid ($100 \mu\text{Ci ml}^{-1}$) for 90 min, washed twice with glucose-saline PIPES buffer (as Fig. 1 legend) and stimulated with Ag in the presence of 0.5 mM 4-deoxy pyridoxine and 0.1 mM L-canaline to prevent breakdown of S1P by S1P aldolase. Lipids were then extracted and analysed by two-dimensional TLC. [^{32}P]S1P was identified by running samples together with S1P standard. Quantification was performed exactly as for the SK assay.

kinase activation mediated by FcεRI, RBL-m1 cells preloaded with anti-DNP IgE were incubated with DHS for 3 min and were stimulated or not with DNP-HSA for 15 s. After cell lysis, the *syk* tyrosine kinase and tyrosine-phosphorylated proteins were immunoprecipitated with anti-*syk* and anti-phosphotyrosine antibodies, respectively, and the immunoprecipitates immunoblotted with anti-phosphotyrosine antibodies (Fig. 3*d*). In these conditions, the effect of DHS on tyrosine phosphorylation of *syk* and other proteins, including the FcεRI β -chain, which was known to be dependent on *lyn* activation¹⁶, was negligible.

Finally, we attempted to connect the FcεRI-mediated SK activation with the activation of tyrosine kinases. The tyrosine kinase inhibitor genistein inhibited SK activation, albeit not completely (data not shown). However, the partial inhibition of SK by genistein did not result in a decrease of S1P formation *in vivo* or in an inhibition of calcium mobilization. Preliminary data suggested that the rate-limiting step in S1P formation was the availability of sphingosine, and that only a small fraction of SK activity was necessary for maximal S1P production. Because genistein only elicited partial inhibition, the residual SK activation could be sufficient to generate a nearly normal S1P response and calcium flux. Therefore the SK pathway might indeed lie downstream of tyrosine kinases, but more investigation would be required to resolve this issue satisfactorily.

We have demonstrated that FcεRI activates an SK enzyme and mediates the production of S1P, which can induce the release of calcium from intracellular stores. In addition, we have provided evidence of specific competition of SK activity and inhibition of S1P formation by DHS after FcεRI triggering. Concomitantly, the

FIG. 3 S1P increases cytosolic free calcium, which is not inhibited by pretreatment with EGTA or DHS (a), and DHS selectively inhibits DNP-HSA (Ag)-induced calcium responses (b) without affecting production of InsP_3 (c) or Ag-induced syk phosphorylation (d). Calcium transient was measured as described in Fig. 1. a, Cells were stimulated either with vehicle (ethanol: methanol, 2:3, 20 μl) or 25 μM S1P (left). EGTA (1.5 mM) was added 20 s before the stimulation with S1P (middle) or cells were pretreated with 25 μM DHS 4 min before the stimulation with S1P (right). b, Cells were pretreated with 25 μM DHS for 4 min before the stimulation with 100 ng ml^{-1} Ag and then stimulated with 100 μM carbachol (CBC). Traces are representative of 3 or more experiments. Inset, cells were preincubated with 25 μM DHS for 4 min before the stimulation with 100 ng ml^{-1} Ag for 1.5 min and production of S1P was measured. Values are expressed as proportional increase compared to the amount of S1P in unstimulated cells; mean $\pm$ s.d. c, Cells were preincubated with DHS (25 μM) for 10 min and stimulated with either Ag (1 $\mu\text{g ml}^{-1}$) for 5 min or CBC (1 mM) for 15 s. Production of InsP_3 was measured as described in Fig. 1. Values are mean $\pm$ s.e.m. from 3 separate experiments performed in duplicate cultures of cells. d, Cells (10^7 cells for immunoprecipitation (IP) with anti-syk antibody or 5×10^6 cells for immunoprecipitation with anti-phosphotyrosine antibody) were loaded with anti-DNP IgE overnight and collected, washed twice with the glucose-saline buffer described in Fig. 1, preincubated for 3 min in the presence or absence of 25 μM DHS and triggered (T) or not (NT) with 100 ng ml^{-1} Ag for 15 s. Incubation was stopped by adding $5 \times$ ice-cold lysis buffer, containing (in mM) 2.5% Triton X-100, 150 NaCl, 200 boric acid (pH 8.0), 25 EDTA, 25 NaF, 5 Na_3VO_4 , 50 $\mu\text{g ml}^{-1}$ aprotinin and pepstatin A, and 25 $\mu\text{g ml}^{-1}$ leupeptin. Lysates were left on ice for 15 min and centrifuged for 30 min at 14,000 r.p.m. in a microcentrifuge at 4°C to remove cell nuclei. Immunoprecipitations, electrophoresis and immunoblotting were performed as described¹⁶. Immunoprecipitations were performed with either a polyclonal rabbit anti-rat syk antibody or the monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology).



$\text{Fc}\epsilon\text{RI}$ -mediated calcium signal is inhibited. Together with the absence of DHS effect on InsP_3 formation, tyrosine kinase activation, and the mIR-mediated calcium pathway, these data indicate that $\text{Fc}\epsilon\text{RI}$ uses the SK pathway to mobilize calcium. □

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Ca^{2+} /S100 regulation of giant protein kinases

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PROTEIN phosphorylation by protein kinases plays a central regulatory role in cellular processes and these kinases are themselves tightly regulated¹. One common mechanism of regulation involves Ca^{2+} -binding proteins (CaBP) such as calmodulin (CaM)². Here we report a Ca^{2+} -effector mechanism for protein kinase activation by demonstrating the specific and $>1,000$ -fold activation of the myosin-associated giant protein kinase twitchin by Ca^{2+} /S100A1₂. S100A1₂ is a member of a large CaBP family that is implicated in various cellular processes, including cell growth, differentiation and motility, but whose molecular actions are largely unknown³. The S100A1₂-binding site is a part of the autoregulatory sequence positioned in the active site that is responsible for intrasteric autoinhibition of twitchin kinase; the mechanism of autoinhibition based on the crystal structures of two twitchin kinase fragments is described elsewhere⁴. Ca^{2+} /S100 represents a likely physiological activator for the entire family of giant protein kinases involved in muscle contractions and cytoskeletal structure^{2,5–9}.

The myosin-associated giant proteins twitchin, projectin and

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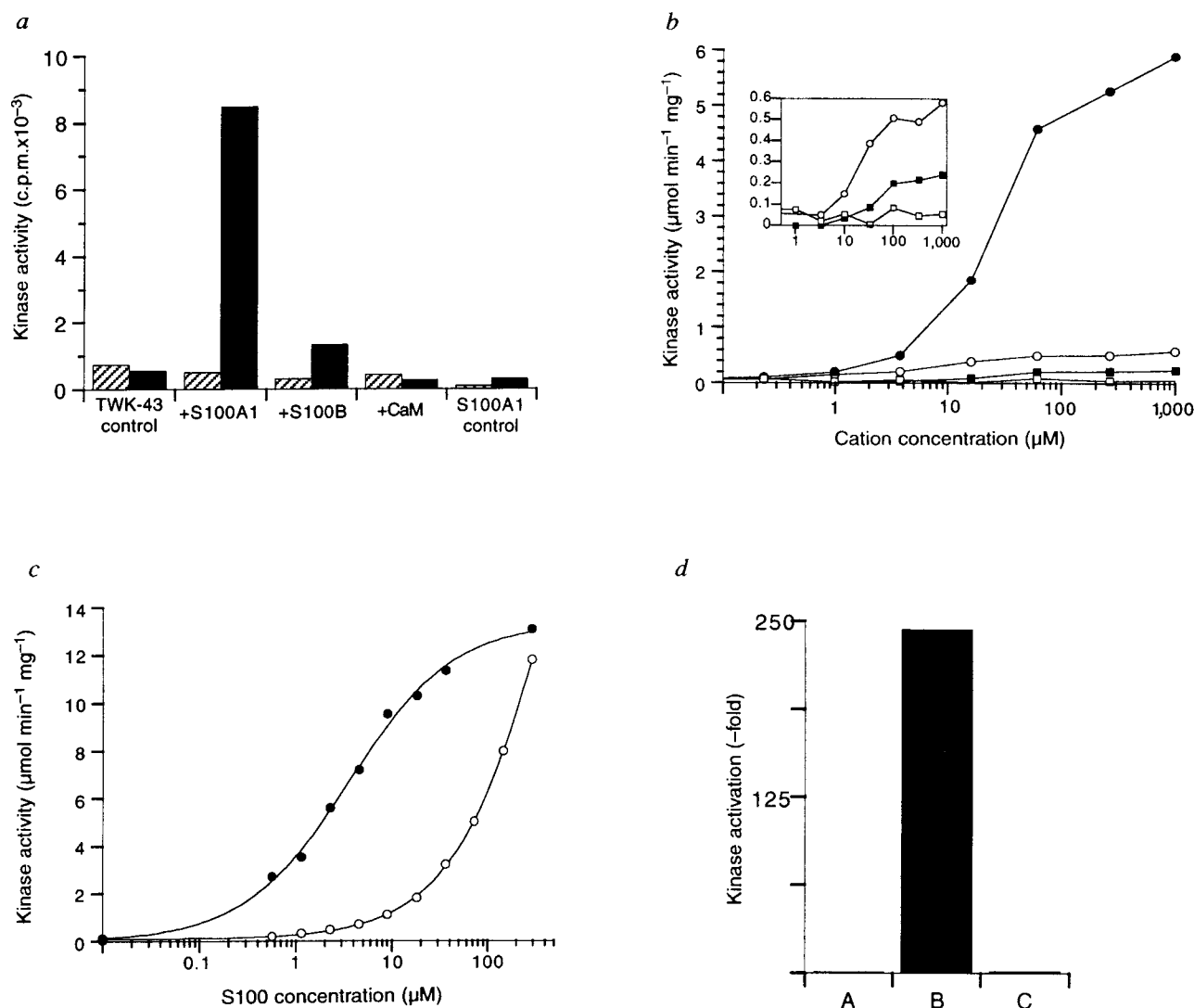


FIG. 1 Activation of twitchin kinase by S100A1₂. *a*, Peptide substrate KKRARAATSNVFA phosphorylation (10 μ M) by TWK-43 (1.2 $\text{ng } \mu\text{l}^{-1}$; expressed and purified as described¹¹) in the presence or absence of CaBPs (2.1–2.4 μ M; determined with the BCA kit (Pierce) and BSA as a standard; proteins from Sigma) and either 0.5 mM CaCl₂ (solid bars) or 1 mM EGTA (hatched bars). *b*, Cation dependence of twitchin kinase (48 $\text{pg } \mu\text{l}^{-1}$) activation by S100A1₂ (2.4 μ M) using 20 μ M peptide substrate. CaCl₂ in the presence of S100A1₂, open circles; CaCl₂ in the presence of S100A1₂ and 10 μ M ZnCl₂, filled circles; ZnCl₂ in the absence of S100A1₂, open squares; ZnCl₂ in the presence of S100A1₂, filled squares. Insert shows three of the curves on a different scale. *c*, Dose–

response curve of TWK-43 (120 $\text{pg } \mu\text{l}^{-1}$) activation by S100A1₂ in the presence of 0.5 mM CaCl₂ (open circles) or 0.5 mM CaCl₂ plus 10 μ M ZnCl₂ (filled circles) using 20 μ M peptide substrate. *d*, Protease control. TWK-43 (48 $\text{pg } \mu\text{l}^{-1}$, A–C) and S100A1₂ (20 μ M, B and C) were first incubated for 4 min at 30 °C in the presence of 0.5 mM CaCl₂ and 10 μ M ZnCl₂. Subsequently, EGTA (2 mM) was added to C, and all kinase reactions were incubated at 30 °C for 4 min using 20 μ M peptide substrate and 400 μ M ATP. TWK-43 activity was stimulated ~250-fold in the positive control reaction containing Ca²⁺/Zn²⁺/S100A1₂ (A, B) but reduced to nearly basal levels in the reaction containing EGTA (C). Phosphorylation reactions were performed as described¹¹ with less than 10% substrate consumed.

titin (relative molecular masses ranging from 700K to over 3,000K) are characterized by multiple repeats of immunoglobulin-like and fibronectin type III-like domains, and a single auto-inhibited protein kinase domain located near the C terminus^{5,6,8,10}. These kinases are most closely related to Ca²⁺/CaM-dependent protein kinases such as myosin-light chains kinases¹; a catalytic fragment of *Aplysia* twitchin phosphorylates myosin light chains¹¹. Although CaM binds to *Aplysia* twitchin^{11,12} and human titin¹³, it does not activate these kinases^{11,13,14} and the mechanism of twitchin activation has been a conundrum. We tested the effect of S100 proteins which occur at high levels with the giant proteins in striated muscle^{3,15}. The autoinhibited *Aplysia* twitchin kinase fragment TWK-43 was activated by S100A1₂ (formerly known as S100($\alpha\alpha$)¹⁶) but not by CaM in a Ca²⁺-dependent manner (Fig. 1*a*). Activation was much lower with the S100B₂ isoform which shares 60% identity with S100A1₂ (ref. 16); TWK-43 or S100A1₂ alone

had no significant protein kinase activity. Low (sub-physiological) concentrations of Zn²⁺, which binds to S100A1 (ref. 17), greatly enhanced the Ca²⁺/S100A1₂-dependent kinase activation (Fig. 1*b*) by shifting the activation curve to ~30-fold lower concentration of S100A1 ($K_{\text{S100A1}_2} = 3.5 \mu\text{M}$; Fig. 1*c*). The twitchin kinase activation depended on the presence of Ca²⁺ and Zn²⁺ during the phosphorylation reaction and did not result from proteolytic generation of constitutively active kinase molecules (Fig. 1*d*). Twitchin kinase activation by 20 μ M S100A1₂ (in the presence of 10 μ M ZnCl₂, 500 μ M CaCl₂) was mainly the result of a more than 1,000-fold increased V_{max} (from 0.01 to 15.6 $\mu\text{mol min}^{-1} \text{mg}^{-1}$), with less than a twofold decrease in K_{peptide} (12.2 to 7.7 μM) and K_{ATP} (38.5 to 22.2 μM) values (data not shown).

To identify the S100A1₂-binding site, we used biotinylated S100A1₂ in overlay assays. S100A1₂ bound only to the autoinhibited kinase fragment TWK-43 in a Ca²⁺/Zn²⁺-dependent manner,

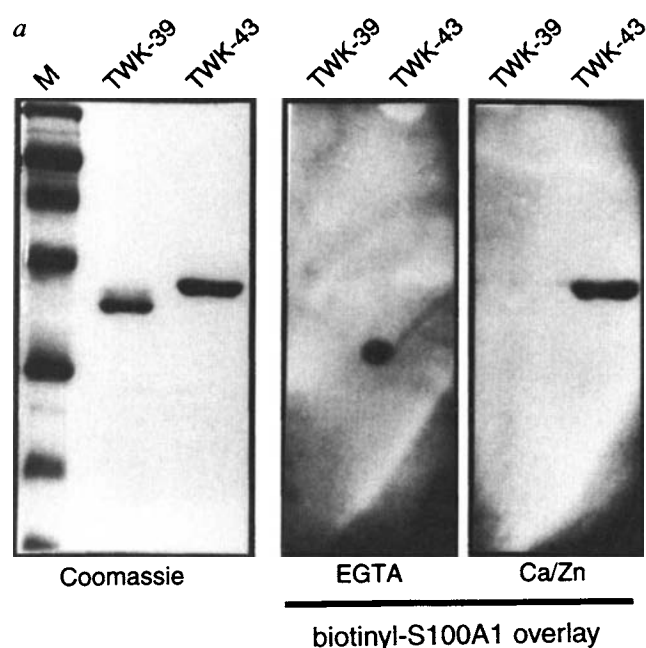


FIG. 2 Identification of the S100A1₂-binding site of twitchin kinase. *a*, Blot-overlay analysis of biotinyl-S100A1₂ (6.5 μM) binding to twitchin fusion proteins TWK-39 (constitutively active; expressed and purified as described¹¹) and TWK-43 (autoinhibited) in the presence of 1 mM CaCl₂ plus 10 μM ZnCl₂ or 5 mM EGTA as indicated (right panels). Also shown is a Coomassie-blue-stained gel of the fusion proteins and molecular weight standards (in thousands; from top to bottom: 200, 97, 66, 45 and

31) (left panel). All samples containing 1 μg protein per lane were separated by 12% SDS-PAGE in the same gel. One part was stained using Coomassie blue, the other parts were transferred onto polyvinylidene difluoride membranes and subsequently used for the overlay analysis. S100A1₂ (500 μg) was biotinylated using biotin-X-NHS (Calbiochem) according to the supplier's instructions. Overlay assays of the membranes with biotinyl-S100A1 were performed as described previously for biotinylated CaM¹². The spot in the centre of the EGTA membrane is located between lanes and does not correspond to a protein band. *b*, Effects of synthetic peptides from the autoinhibitory region (0.2 μg μl⁻¹ (53.6 μM) TWC1: LKGDHSNLTSRIPSSRYNKIRQKI-KEKYADW; 0.1 μg μl⁻¹ (44.9 μM) TWC2: SRYNKIRQKIKEKYADW)¹² on TWK-39 (24 μg μl⁻¹, hatched bars) and TWK-43 (120 μg μl⁻¹, filled bars) activity in the presence of 500 μM CaCl₂, 10 μM ZnCl₂, 12 μM S100A1₂ (as indicated), 20 μM peptide substrate, and 400 μM ATP. Values represent the mean ± standard error of three experiments.

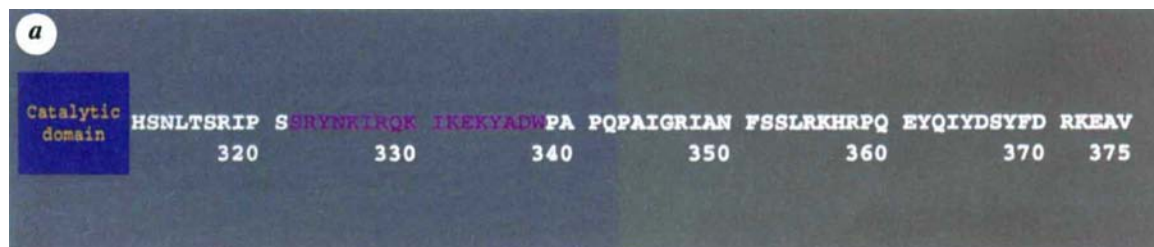
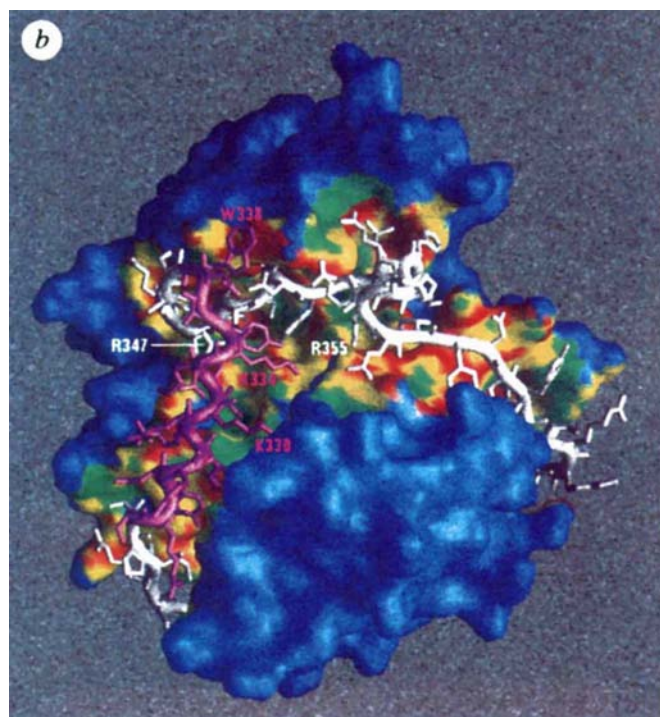


FIG. 3 Structure of the S100A1₂-binding site of the *Aplysia* twitchin kinase. *a*, Schematic representation of TWK-43 and the autoinhibitory sequence¹¹. The S100A1₂-binding site is indicated. *b*, Structure of TWK-43. The molecular surface²⁵ of the catalytic kinase domain is coloured according to its shape complementarity²⁶ with the autoinhibitory sequence (red, $S_c > 0.76$; yellow, $0.76 > S_c > 0.3$; green, $0.3 > S_c > -0.3$; light blue, $S_c > -0.3$; $S_c = 1$ for perfect complementarity, and $S_c = 0$ for no surface complementarity). The S100A1₂-binding site is coloured magenta; the rest of the autoinhibitory sequence is white. Highly conserved residues in autoinhibitory sequences of Ca²⁺-regulated protein kinases⁴ are labelled. The autoinhibitory sequence follows the same path in TWK-43 and in the structure of an extended fragment of twitchin containing the C-terminal Ig-like domain⁴. A detailed account of both these crystal structures, the autoinhibitory mechanism and the structural properties of Ca²⁺/S100A1₂ regulation are given elsewhere⁴.



but not to the shorter TWK-39 fragment lacking the autoinhibitory sequence (Fig. 2a). Furthermore, the synthetic peptide TWC1 from the autoinhibitory sequence¹¹ inhibited S100A1₂ activation of TWK-43 by 73%, but the constitutively active kinase TWK-39 (whose activity was not enhanced by S100) by less than 10%; similar results were obtained with the related peptide TWC2 (Fig. 2b). The stronger inhibitory effect of these peptides on kinase activation than on kinase activity suggests that this twitchin sequence encompasses the S100A1₂ binding site. The site is therefore located in a position similar to CaM-binding sites in Ca²⁺/CaM-regulated protein kinases^{1,4}.

To understand the regulatory role of the S100A1₂-binding site at the molecular level, we determined the crystal structure of TWK-43 at 2.3 Å resolution (Fig. 3 and ref. 4). The autoinhibitory contacts in TWK-43 exploit essentially all prominent features of the active site, including binding sites for protein substrate, ATP and Mg²⁺, the catalytic residues and the Thr-rich activation loop¹⁸. The major part of the proposed S100A1₂-binding site forms a basic-amphipathic helix, a structural feature inferred in CaM-binding sites^{19–21}. This similarity explains why CaM and S100A1₂ can bind to the same site in twitchin (Fig. 2 and ref. 12). However, the selective activation by S100A1₂ but not CaM suggests different modes of binding, probably resulting from the different topologies of their Ca²⁺-binding domains^{22,23}. The extensive contacts between the autoinhibitory sequence and the active site are consistent with the predominant $V_{\max}$ effect of S100-dependent twitchin kinase activation, and explains why excess substrates (ATP and peptide) alone cannot reverse the autoinhibition.

Our results reveal a novel Ca²⁺-effector mechanism for protein kinase activation. The high catalytic activity of the stimulated twitchin kinase even at limiting Zn²⁺ and S100A1₂ concentrations underscores the significance of S100A1₂ activation. Because the twitchin kinase represents the first enzyme whose activity is dramatically enhanced by a member of the S100 family, other proposed S100 functions³ could be mediated by as-yet unidentified protein kinases⁴. The autoinhibited *Caenorhabditis elegans* twitchin kinase can also be activated by Ca²⁺/S100A1₂ (our unpublished data), indicating that this mechanism is not restricted to the *Aplysia* enzyme. The S100A1₂-concentration required for twitchin kinase activation is well within the physiological range of tissues such as human heart where S100A1₂ comprises 0.2% (by mass) of the total protein¹⁵. It seems reasonable that cardiac titin will also be regulated by Ca²⁺/S100A1₂, because the cardiac titin sequence corresponding to the twitchin S100A1₂-binding site also binds CaM, albeit without activation¹³. The Ca²⁺ concentration required for half-maximal kinase activation (~20 µM) is similar to the cytosolic Ca²⁺ concentration in stimulated skeletal muscle; moreover, the local Ca²⁺ concentration close to the release sites in the sarcomere is likely to be considerably higher, as recently demonstrated by modern imaging techniques²⁴. The identification of S100 as an activator of twitchin kinase provides an experimental system for future studies of S100/target protein interactions and, together with our structural studies⁴, brings us closer to a comprehensive understanding of the molecular choreography of the activation process of giant protein kinases □

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Altered circadian activity rhythms and sleep in mice devoid of prion protein

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THERE is a wealth of data supporting a central role for the prion protein (PrP) in the neurodegenerative prion diseases of both humans and other species¹, yet the normal function of PrP, which is expressed at the cell surface of neurons and glial cells^{2,3}, is unknown. It has been speculated that neuropathology may be due to loss of normal function of PrP (ref. 4). Here we show that in mice devoid of PrP there is an alteration in both circadian activity rhythms and sleep patterns. To our knowledge, this is the first null mutation that has been shown to affect sleep regulation and our results indicate that the pathology of at least one of the inherited prion diseases, fatal familial insomnia⁵, where there is a profound alteration in sleep and the daily rhythms of many hormones^{6–10}, may be related to the normal function of the prion protein.

Mice devoid of the prion protein PrP^C develop and reproduce normally^{11,12} and show no impairment in learning ability^{12,13}. Recently, however, abnormalities of GABA-mediated inhibitory neurotransmission and weakened long-term potentiation were reported in the hippocampus of PrP-null mice^{4,13}. These results suggested that PrP may be involved in synaptic transmission and that neuropathology of the prion diseases may result from a loss of function of PrP^C, rather than from the build-up of the pathological prion protein PrP^{Sc} during disease. We now provide evidence that PrP may also be involved in the regulation of sleep and that loss of this function may result in neurodegeneration in one of the prion diseases, fatal familial insomnia.

Investigation of the circadian system resulted in highly significant differences in period length of the activity rhythm between mice lacking PrP (*Prn-p*^{0/0} mice) and wild-type mice (Fig. 1). The

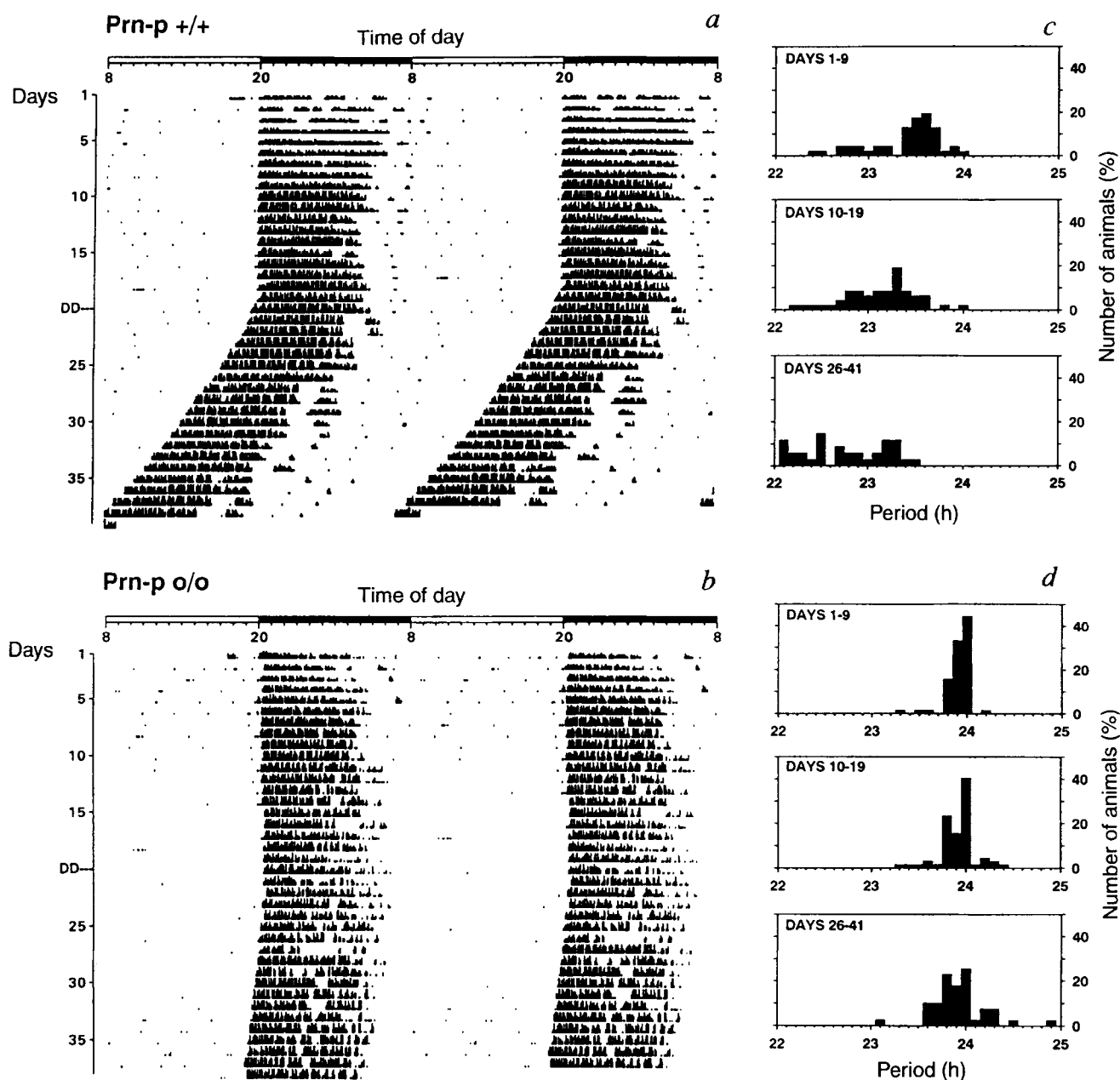


FIG. 1 Motor activity rhythms (running wheel revolutions) in light-dark (L-D) conditions and in constant darkness (D-D). Activity records of representative mice, double plotted (as shown by the time axis). The timing of the L-D cycle (12 h of light: 50–130 lux) is indicated by the black and white bar at the top. After recording activity under entrained conditions for 19 d, the mice were released into D-D. *a*, Record of a homozygous wild-type mouse (*Prn-p*^{+/+}) with a period of 23.3 h. *b*, A null mouse (*Prn-p*^{0/0}) which maintained an activity period of only slightly less than 24 h (23.9 h). *c*, *d*, Frequency distribution of circadian period for 9–16 d intervals in D-D for *Prn-p*^{+/+} (*n* = 46, 47 and 35) and *Prn-p*^{0/0} (*n* = 63, 64 and 44). METHODS. The motor activity rhythm of *Prn-p* null mice (*Prn-p*^{0/0}) and wild-type mice (*Prn-p*^{+/+}) was recorded. Both genotypes had a mixed genetic background derived from 129/Sv and C57BL/6J mice¹². Activity rhythms

were measured in a L-D cycle (at least 12 d) to assess synchronization and entrainment to the zeitgeber, and in D-D (age 7–26 wk at D-D onset) to determine the endogenous circadian period. Mice were individually kept in cages equipped with a running wheel, provided with water and food *ad libitum* and placed in a ventilated room at 21 °C. Running-wheel revolutions (all cages) and infrared activity (20 cages with sensors mounted above the cage), were recorded on-line and stored in 1-min bins (Stanford Software Systems, Chronobiology Kit). The period of the activity rhythm was determined by χ^2 periodogram analysis (Stanford Software Systems, Chronobiology Kit) on 6-min bins for several intervals in D-D: days 1–9, 10–19, 26–35 or 26–41 or 32–41 and 42–51. No differences in period were observed between running wheel and infrared data.

Prn-p^{0/0} mice exhibited the longest period, with values close to 24 h (23.9 h $\pm$ 0.02; range 23.3–24.4 h) in the second 10-d interval in constant darkness (D-D). In the 26–41-day interval in D-D the mean circadian period of *Prn-p*^{0/0} and *Prn-p*^{+/+} mice differed by 66–84 min (Fig. 2), a large difference considering the maximum differences of 25 and 59 min found in up to 12 inbred mouse strains^{14,15}. The scatter of activity period values in *Prn-p*^{0/0} was smaller than that in *Prn-p*^{+/+} mice (Fig. 1c, d).

To determine the specificity of the lack of the *PrP* gene and its product on the results, the possibility of rescuing the phenotype in transgenic (Tg) mice¹⁶ was tested. The period of the activity rhythm in intervals 1–4 in Tg mice were significantly shorter than in the null mice, and longer than in *Prn-p*^{+/+} mice (Fig. 2).

Prolonged recordings in D-D for up to 51 d (*n* = 13–16 mice) and 112 d (*n* = 8–14 mice) revealed a remarkable stability of the period in the null mice. In contrast, the period of *Prn-p*^{+/+} mice

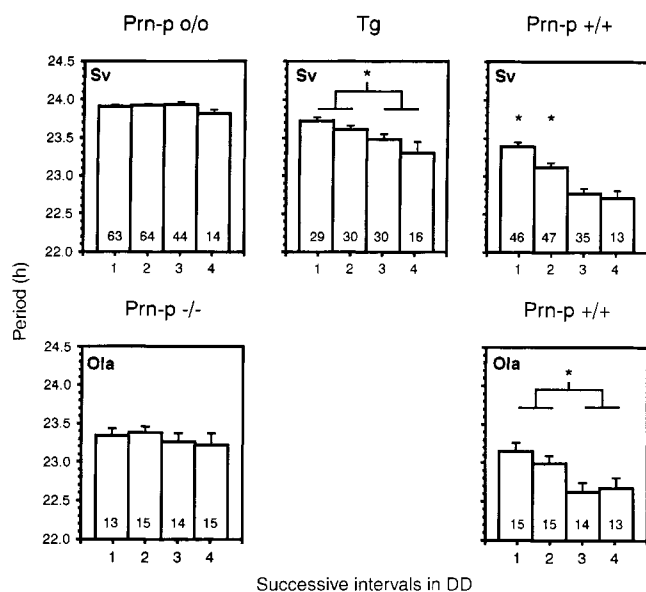


FIG. 2 Comparison of the progressive changes in the period of locomotor activity during constant darkness (D-D) in two strains of mice, 129/Sv (top) and 129/Ola (bottom). Mice devoid of PrP ($Prn-p^{0/0}$, $Prn-p^{-/-}$) and wild-type ($Prn-p^{+/+}$) of both strains, and transgenic mice (Tg) overexpressing the prion protein¹⁶. Bars 1–4 represent the mean circadian locomotor activity period and s.e.m. of consecutive intervals in D-D (days 1–9, 10–19, 10 d between days 14–41, 42–51; number of animals is shown within the bars). Two-way ANOVA within each strain, factors 'genotype': $P < 0.0001$ and 'interval in D-D': $P < 0.01$; one-way ANOVA factor 'interval in D-D', $Prn-p^{0/0}$, $Prn-p^{-/-}$: $P > 0.4$; Tg, $Prn-p^{+/+}$: $P < 0.008$. Asterisks: within each strain, intervals which differed significantly from all other intervals or, if joined by brackets, from pairs of intervals (Duncan's multiple range test, $P < 0.05$). The results did not differ when only the mice recorded for 51 d in D-D were included.

METHODS. Tg mice expressed high levels of PrP protein from a minigene construct (tga20/+; ref. 16), which showed higher brain PrP levels than did $Prn-p^{+/+}$ mice. The presence of PrP mRNA in the brain¹⁶, including the SCN (unpublished results) was confirmed by *in situ* hybridization in both genotypes. 129/Ola mice were inbred mice with a complete absence of both PrP and its mRNA¹¹ ($Prn-p^{-/-}$), and 129/Ola $Prn-p^{+/+}$, the corresponding wild type.

became more variable, shortened progressively, and stabilized after 20 d. This result in the wild-type mice is consistent with data obtained in mice and rats^{17,18}. Also, the Tg mice exhibited a progressive shortening of the activity period and thereby resembled wild-type more than null mice. As the conditions before the release of the animals into D-D can affect the subsequent period, the differences we observed in D-D may have been influenced by a different susceptibility of the PrP-null mice to the prior lighting conditions, and may be clarified in mice reared in D-D. On the other hand, the phase delays or advances elicited by a 1-h light pulse (~ 450 lux) at circadian time (CT) 14.1 ± 0.2 and CT 0.4 ± 0.1 was similar to the results obtained at the same CTs in other mice, including albino strains^{15,19}, and did not differ between genotypes (null mice: -2.2 ± 0.3 , $n = 14$; 0.5 ± 0.1 , $n = 13$; wild type: -2.7 ± 0.8 , $n = 13$; 0.9 ± 0.7 , $n = 9$; and Tg: -2.5 ± 0.3 , $n = 13$; 0.001 ± 0.3 , $n = 13$). Cellular interactions might be necessary to couple individual cells of the suprachiasmatic nuclei (SCN), and PrP^C, a membrane-surface protein, might contribute to this mechanism.

None of the other activity variables differed between the genotypes within a strain. Thus the mice did not differ in the synchronization to the L-D cycle (Fig. 1): wheel-running activity began immediately after the lights went out, and upon release into D-D, the phase of entrainment (the beginning of activity onset in D-D relative to the last days in L-D, 12:12), was similar in null mice and wild type. Also, the daily activity counts in L-D and in D-D, and the L-D ratio of activity counts were similar among the genotypes. Therefore the differences in period were not merely a consequence of different levels of motor activity. The activity profile in the L-D condition was a further discriminating feature of null mice. Both $Prn-p^{+/+}$ and Tg mice were significantly more active in the first half of the dark period than $Prn-p^{0/0}$ mice, whereas an inverse relationship prevailed in the second half ($P < 0.0001$; *t*-test). No differences were present between $Prn-p^{+/+}$ and Tg, so that also this feature of the $Prn-p^{0/0}$ activity rhythm was restored by the introduction of $Prn-p$ in Tg mice (mean activity counts in the first half versus those in the second half of the dark period: $Prn-p^{+/+}$: 303.9, 88.6; $Prn-p^{0/0}$: 273.3, 121.6; Tg: 312.8, 80.3). Thus, overexpression of the PrP in Tg mice fully restored some aspects of circadian rhythms, whereas others remained intermediate between $Prn-p$ -null and wild-type mice. Even though the transgene restored PrP^C expression in the SCN, ectopic expression or altered levels of PrP^C expression may lead to an incomplete rescue of the phenotype.

As the mixed genetic background may have affected the results, the period of locomotor activity was determined in another inbred

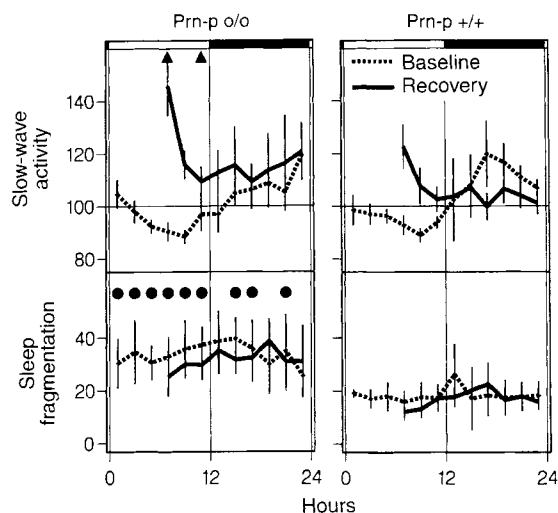


FIG. 3 Sleep fragmentation and slow-wave activity (SWA; mean EEG power density in the 0.75–4.0 Hz band) for a 24-h baseline and the subsequent day after 6 h sleep deprivation (SD)²⁷ in null mice and wild type ($Prn-p^{0/0}$, $Prn-p^{+/+}$). Sleep deprivation began at light onset and was performed by gentle handling²⁶. SWA in non-REM sleep (NREMS) is expressed relative to the 24-h baseline mean (100%) per animal, and sleep fragmentation as the number of brief awakenings per hour of NREMS (single events within a 4-s epoch or a waking episode lasting < 16 s)²⁷. Mean 2-h values $\pm$ s.e.m. ($n = 8$ for each genotype). The null mice exhibited more waking and less NREMS in the second half of the dark period than wild type and their cortical temperature (T_{CRT}) was higher (mean values as percentage of 12 h, null mice versus wild type; waking 63.9 ± 4.3 , 52.0 ± 2.9 ; NREMS: 30.0 ± 3.5 , 41.0 ± 2.3 ; T_{CRT} : $36.7^\circ \text{C} \pm 0.1$, 35.9 ± 0.2 ; $P < 0.04$ for waking, NREMS and T_{CRT} , 2-tailed *t*-test). The higher T_{CRT} reflected the higher amount of waking, confirming the differences obtained in the activity profile. Triangles: significant differences of the effects of s.d. between the two genotypes $P < 0.05$, *t*-test (two-way ANOVA factor 'genotype', '2-h interval', $P < 0.001$ for both factors); filled circles: differences between the baselines of the two genotypes, $P < 0.05$, *t*-test (two-way ANOVA factors 'genotype' and '2-h interval' for baseline sleep fragmentation: $P < 0.0001$ for both factors). The genotypes differed in the response to s.d. in all 6-h recovery intervals ($P < 0.04$, *t*-test).

METHODS. Adult PrP-null mice and wild-type controls²⁵ ($Prn-p^{0/0}$ and $Prn-p^{+/+}$; mean age 80.6 ± 4.3 d) were implanted with chronical electrodes for EEG and EMG recordings and with a cortical thermistor to record T_{CRT} ^{26–28} and adapted to the light–dark cycle (12:12 h 150 lux) for 3 weeks before recordings. For the signal analysis and scoring of the vigilance states and brief awakenings, see refs 26–28.

mouse strain, 129/Ola. Mice devoid of PrP ($Pm-p^{-/-}$) and the corresponding wild type ($Pm-p^{+/+}$) were compared¹¹. Prolonged recording in D–D confirmed the main aspects of the previous results (Fig. 2): the period of the null mice was significantly longer than that of the wild type and remained unchanged in the course of the recording, contrasting the shortening of the period in Ola strain $Pm-p^{+/+}$, which reached, as in the Sv strain, a stable level after 20 d in D–D. The differences in period between the Ola and Sv strains did not affect the differences observed between the null mice and wild type within each strain.

Cresyl fast violet histochemistry and immunohistochemistry of the SCN with antiserum against vasoactive intestinal polypeptide (VIP; $n = 4$ mice per genotype, 129/Ola) revealed no obvious morphological differences and a similar distribution and degree of labelling of VIP protein in both genotypes.

$Pm-p^{0/0}$ and $Pm-p^{+/+}$ also differed in their sleep structure and the response to sleep deprivation (Fig. 3). Although the 24-h values of the vigilance states did not differ between the genotypes, non-rapid eye movement sleep and waking distribution in the dark period differed. Moreover, sleep fragmentation was more prominent in $Pm-p^{0/0}$ mice (Fig. 3) and they showed a much larger response to sleep deprivation than the $Pm-p^{+/+}$. The increase of electroencephalogram (EEG) slow-wave activity (SWA) during recovery sleep was almost twice as large in $Pm-p^{0/0}$ than in $Pm-p^{+/+}$ (Fig. 3).

The genetic mechanisms underlying sleep regulation are unknown, despite early efforts to characterize the inheritance of activity and sleep patterns^{20–22}. It is well established that sleep in mammals is regulated as a function of prior wakefulness, as indicated by the predictable change of SWA^{23,24}. Our results are evidence for a null mutation that affects sleep regulation, and show specifically that PrP^C, a well conserved protein present in many mammalian species²⁵, may be involved in maintaining sleep continuity or regulating sleep intensity.

In summary, our results show that loss of PrP affects the circadian activity rhythm and sleep. As the two lines of PrP-null mice were produced by different targeting strategies, our results demonstrate that this phenotype is due to a loss of PrP and is not an artefact of the gene-targeting techniques. Because of the intriguing similarities to rhythm and sleep alterations in fatal familial insomnia, we suggest that loss of function of PrP leads to neurological dysfunction in at least one of the prion diseases. □

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Control of cAMP-regulated enhancers by the viral transactivator Tax through CREB and the co-activator CBP

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THE Tax protein of human T-lymphotrophic virus (HTLV)-1 activates expression of the HTLV-1 long terminal repeat through a DNA element that resembles the cellular cyclic AMP-regulated enhancer (CRE)^{1,2}. Tax contains a transcriptional activation domain³, but its ability to activate gene expression depends on interactions with cellular CRE-binding proteins such as CREB. Whether Tax can activate the expression of cellular CRE-containing genes has been controversial. Here we show that Tax can activate both the HTLV-1 and consensus cellular CREs, and propose that this activation may occur through mechanisms that are differentially dependent on CREB phosphorylation. Tax not only increases the binding of CREB to the viral CRE but also recruits the transcriptional co-activator CBP^{4,5} in a manner independent of CREB phosphorylation. In contrast, association of Tax with the cellular CRE occurs through CBP which, in turn, is recruited only in the presence of phosphorylated CREB.

Although the participation of CRE-binding proteins such as CREB in Tax activation of the HTLV-1 long terminal repeat is well established^{6,7}, the mechanisms underlying Tax activation of cellular CRE-containing genes are unclear. Several studies have shown that Tax function requires DNA sequences that are specific to the HTLV-1 promoter^{8–10}, and cellular CRE-containing genes have generally been found to be unresponsive to Tax^{11,12}. Nonetheless, genetic analysis of Tax mutants indicates that cellular CRE-containing genes account for critical aspects of Tax-mediated cell transformation¹³. To compare the ability of Tax to activate cellular and viral CREs, we constructed reporter genes containing either a single copy of the somatostatin CRE¹⁴, as a prototype of a cellular CRE, or the entire HTLV-1 U3 region¹⁵. Transfections were performed in F9 teratocarcinoma cells, which contain CBP but require exogenous CREB and protein kinase A (PKA) for CRE-reporter activation¹⁴. The ability of Tax to augment CREB-mediated induction of the cellular CRE was dependent on CREB phosphorylation, as the augmentation was prevented when PKA was omitted, and was significantly decreased when the consensus PKA site in CREB was mutated (Fig. 1a, b). In contrast, Tax activated expression of the HTLV-1 reporter even in the absence of PKA (Fig. 1c). Similar results were obtained in studies using the promoter proximal HTLV-1 CRE alone (data not shown). These results suggest that Tax activates the cellular and viral CREs through different mechanisms.

We used a fluorescence polarization binding assay^{5,16,17} to examine the effects of Tax on DNA binding. In the absence of Tax, CREB bound poorly to the HTLV-1 CRE ($K_d > 100$ nM; Fig. 2a); addition of Tax lowered the K_d to 6.5 nM. Tax did not alter CREB binding to the cellular CRE, however (K_d 4.5 nM versus 3.2 nM; Fig. 2b). Similar data were obtained using phosphorylated CREB (data not shown). Thus, although Tax can increase the affinity of CREB for the HTLV-1 CRE, Tax activation of the somatostatin CRE must occur through another mechanism.

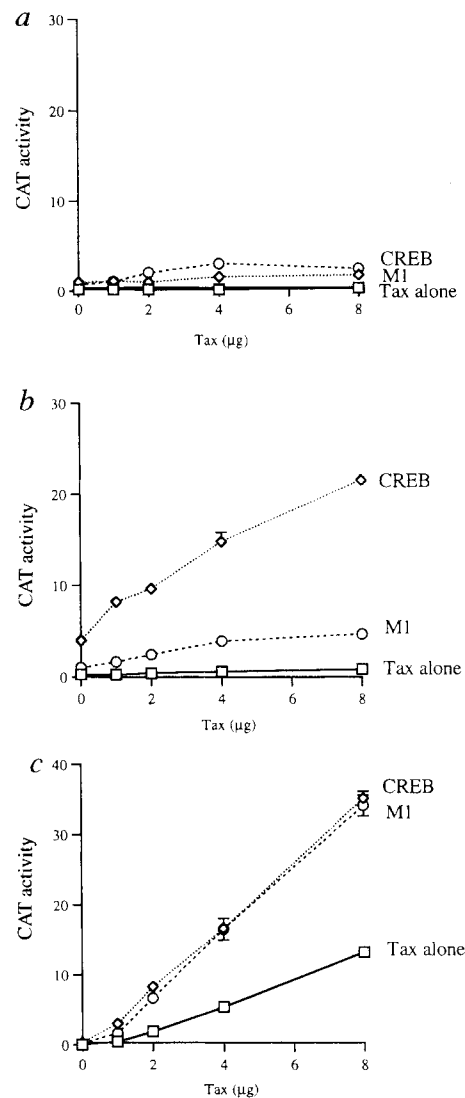


FIG. 1 Responses of the cellular CRE and HTLV-1 reporter genes. **a**, Cellular CRE without PKA; **b**, cellular CRE with PKA; **c**, pU3R without PKA. F9 teratocarcinoma cells were transfected with p(-71) somatostatin-chloramphenicol acetyltransferase (CAT)¹⁴ (**a**, **b**) or pU3R-CAT¹⁵ (**c**) along with Rous sarcoma virus (RSV)-CREB341 (ref. 20) or RSV-CREB1 (in which the PKA phosphorylation site has been mutated to alanine²⁰), RSV-Tax (S. Marriott, unpublished), and RSV-PKA (gift from R. Maurer), as indicated. Results are expressed as CAT activity (mean $\pm$ s.e., $N > 3$), normalized for luciferase activity.

METHODS. **a**, **b**, F9 cells were seeded with 3.5×10^5 cells per plate and were transfected by calcium phosphate precipitation¹² with 4 μ g RSV-PKA as indicated, 2 μ g RSV-luciferase, 0–8 μ g RSV-Tax, 4 μ g RSV-CREB or CREB1, and 5 μ g p(-71) somatostatin-CAT. For **c**, 2 μ g RSV-CREB or CREB1 and 2 μ g pU3R-CAT were used.

Because Tax activation of the cellular CRE depended upon CREB phosphorylation, we considered whether Tax could alter the affinity of the CREB co-activator CBP. In the context of the somatostatin CRE, the binding of CBP to phosphorylated CREB in the presence and absence of Tax was similar ($K_d = 344$ nM and 360 nM, respectively; Fig. 2c). The poor binding of CREB to the HTLV-1 CRE in the absence of Tax precluded accurate measurement of CBP affinity but, in the presence of Tax, the K_d of CBP for the phosphorylated CREB:HTLV-1 CRE complex was 150 nM (Fig. 2e). Surprisingly, Tax also allowed CBP to associate with the complex containing non-phosphorylated CREB, but only in the context of the HTLV-1 CRE ($K_d = 200$ nM; Fig. 2f). Thus, Tax increases the affinity of CREB for the HTLV-1 CRE, increases the affinity of CBP for the HTLV-1 CRE:CREB complex, and renders this latter interaction independent of CREB phosphorylation. In the context of the somatostatin CRE, non-phosphorylated CREB associated very poorly with CBP regardless of Tax (Fig. 2d).

Because Tax activation of the prototypical cellular CRE-containing gene did not appear to result from increasing CBP affinity, we investigated whether Tax could bind to CBP directly. Tax binds *in vitro* to CBP residues 451–682 and the corresponding portion of the CBP homologue p300 (566–663) (Fig. 3a), regions characterized previously as containing the 'CREB binding domain'^{4,16}. Tax

also bound weakly to glutathione S-transferase (GST)-CBP fusion proteins containing residues 1,099–1,331 and 1,680–1,891 (data not shown). To test whether Tax interacts with CBP or p300 *in vivo*, we used yeast two-hybrid and co-immunoprecipitation assays. In the yeast two-hybrid assay, the combination of Lex-CBP(451–682) and VP16 Tax allowed growth of the yeast reporter strain in the absence of histidine and promoted β -galactosidase expression (Fig. 3b). We also demonstrated that p300 associates with Tax in HTLV-1 infected Hu T-102 cells (Fig. 3c). To determine whether this association could augment CBP/p300-mediated transcriptional activation, we tested the ability of a Gal-CBP(451–682) fusion gene to activate a Gal-CAT reporter in F9 teratocarcinoma cells. Tax enhanced the activity of the fusion gene in a dose-dependent manner, while slightly diminishing the activity of a Gal-CBP(1,678–2,441) fusion gene (Fig. 3d).

The hypothesis that CBP interacts with both Tax and phosphorylated CREB at the cellular CRE implies the formation of a quaternary complex. This possibility was tested by using an avidin-biotin complex assay. Complexes containing a biotinylated CRE, phosphorylated or non-phosphorylated CREB, CBP and Tax were collected on avidin beads and assayed for their constituent proteins. Tax associated with the somatostatin CRE only in the presence of phosphorylated CREB and CBP (Fig. 4a). Addi-

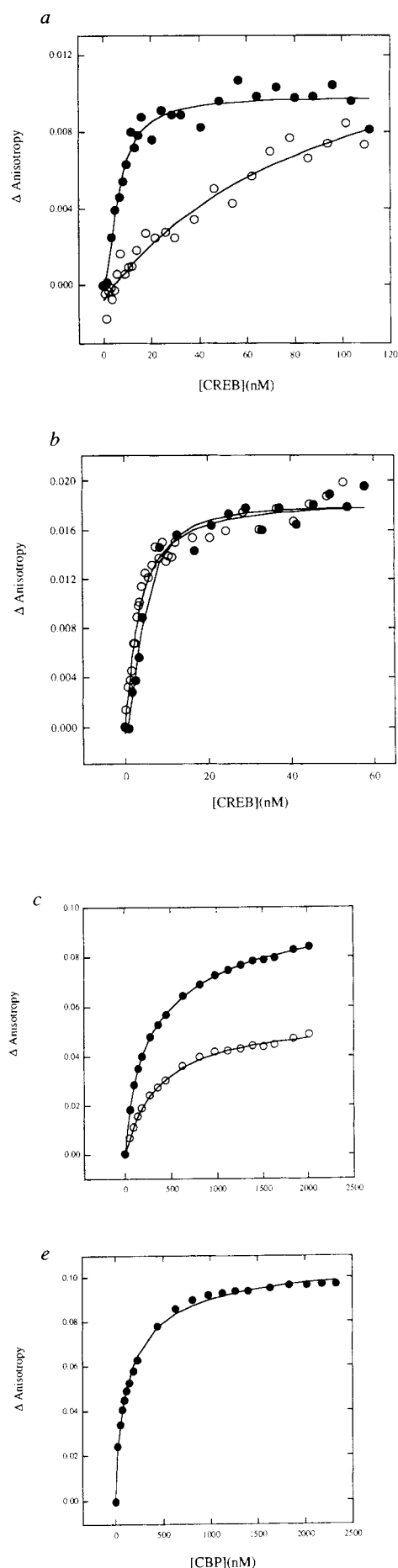
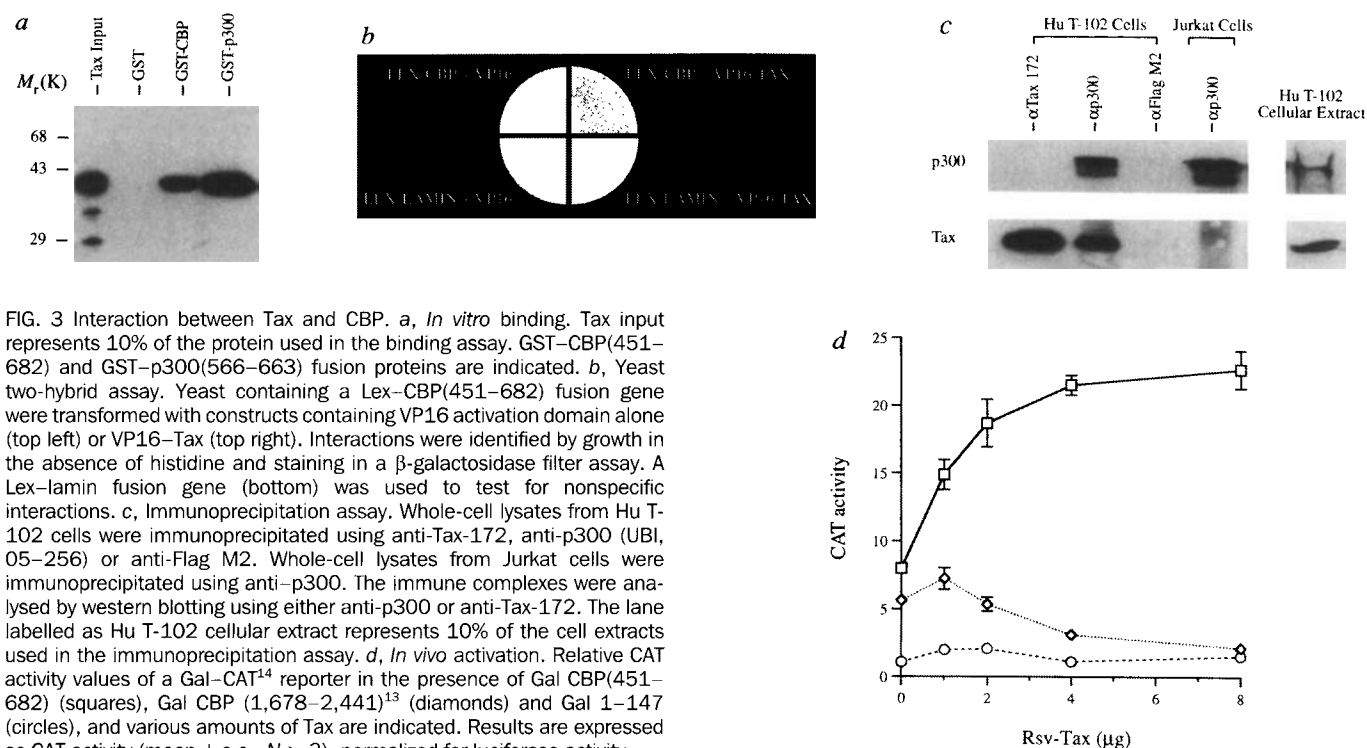


FIG. 2 Effects of Tax on CREB and CBP binding. *a*, Fluorescence anisotropy measurements of CREB binding to the HTLV-1 CRE in the absence (open circles) and presence (filled circles) of 250 nM Tax. *b*, Binding of CREB to the cellular CRE in the presence and absence of Tax. *c*, CBP binding to a phosphorylated CREB:cellular CRE complex in the presence or absence of Tax. *d*, Binding of CBP to the non-phosphorylated CREB:cellular CRE complex. No specific binding was detected in the absence or presence of Tax. *e*, CBP binding to a phosphorylated CREB:HTLV-1 CRE complex in the presence of Tax. *f*, CBP binding to the non-phosphorylated CREB:HTLV-1 CRE complex. In the absence of Tax, there was no specific CBP binding. K_d for CBP in the presence of Tax was 200 nM.

METHODS. A 28-base 5'-fluoresceinated oligonucleotide representing the sense strand of the most proximal HTLV-1 Tax response element (5'-TCCTCAGGCGTTGACGACAACCCCTCAC-3') and a 23-base 5'-fluoresceinated oligonucleotide representing the somatostatin CRE (5'-CCTTGGCTGACGTCAGAGAGAGC-3') were annealed to antisense oligonucleotides. Generation of Tax, phosphorylated CREB, CBP(1-682), and fluorescence polarization measurements were as described^{5,16}. For the CBP binding assays, fluorescein-labelled oligonucleotides (5 nM) were incubated with saturating amounts (30 nM) of non-phosphorylated or phosphorylated CREB and increasing amounts of CBP in the presence or absence of 250 nM Tax.



METHODS. **a**, GST-pulldown assay was performed as described^{5,16}. Tax was detected by using TAB172 monoclonal antibody²¹ and ECL. **b**, DNA encoding CBP(451-682) was cloned into BTM116 (gift from S. Hollenberg). Bait and target vectors were transformed into yeast strain L40 as described²² and were plated onto His⁻ plates supplemented with 15 mM 3-aminotriazole. A β -gal filter assay²³ was used to visualize interactions. **c**, Cell extract

preparation and conditions for immunoprecipitations and immunoblot assay have been described¹⁶. **d**, F9 cells were transfected with Gal-CAT reporter, 4 μ g RSV-PKA, 2 μ g RSV-luciferase, and 0.5 μ g of either RSV-Gal CBP(451-682), RSV-Gal CBP(1,678-2,441) or RSV-Gal 1-147, and 0-8 μ g of RSV-Tax, as indicated.

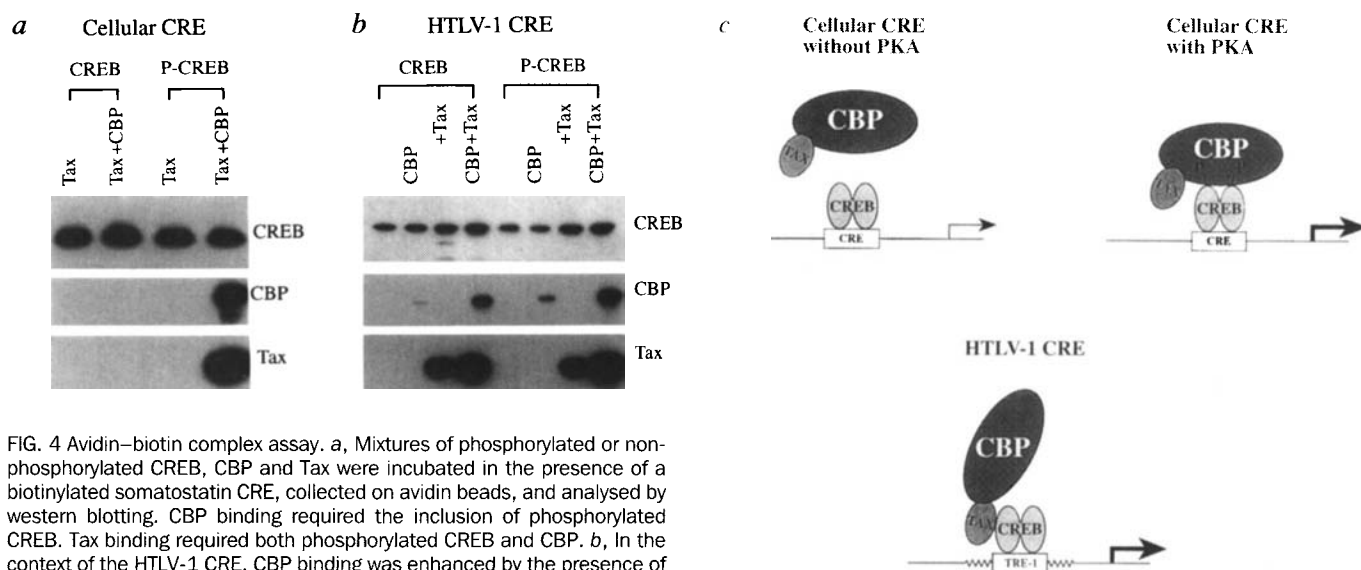


FIG. 4 Avidin-biotin complex assay. **a**, Mixtures of phosphorylated or non-phosphorylated CREB, CBP and Tax were incubated in the presence of a biotinylated somatostatin CRE, collected on avidin beads, and analysed by western blotting. CBP binding required the inclusion of phosphorylated CREB. Tax binding required both phosphorylated CREB and CBP. **b**, In the context of the HTLV-1 CRE, CBP binding was enhanced by the presence of Tax, but Tax association did not require CBP. **c**, Model for Tax, CREB and CBP binding. In the context of the cellular CRE, Tax is recruited through CBP, which requires phosphorylated CREB. In the context of the HTLV-1 CRE, CBP is recruited to the complex regardless of CREB phosphorylation, possibly through interactions with Tax rather than CREB. Tax and CREB depend on each other for DNA binding. Jagged lines represent HTLV-1 CRE sequences proposed to contribute to CREB binding¹⁰.

METHODS. A 28-base oligonucleotide (5'-CGAGCCTTGCTGACGTGACAGAGAGAGCG-3') was annealed with a 36-base oligonucleotide (5'-TCGACGCTCTCTGACGTGACCAAGGCTCGAGCT-3') to form a double-stranded DNA representing the somatostatin CRE and surrounding sequences. A 31-base oligonucleotide (5'-CGAGCCTCAGGCGTTGACGACCAACCCTCAG-3') was annealed to a 39-base oligonucleotide (5'-TCGACT-

GAGGGGTTGCTGCTCAACGCTGAGGCTCGAGCT-3') to form a double-stranded DNA representing the promoter proximal HTLV-1 Tax response element. The 3' ends of both double-stranded DNAs were labelled with biotin-14-dATP as described²⁴. Labelled DNA (200 ng) was incubated with 250 nM CREB or phosphorylated CREB, 250 nM Tax, and 800 nM CBP(1-682) in 200 μ l buffer containing 50 mM Tris, pH 7.6, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 5 mM MgCl₂, 0.1% Triton, 5% glycerol, 2.5 mg ml⁻¹ BSA, 10 μ g ml⁻¹ poly [d(I-C)] for 2 h at room temperature before addition of streptavidin beads (Pierce). Mixtures were then incubated at room temperature for 1 h and washed four times in binding buffer without BSA and poly [d(I-C)]. Proteins were eluted in SDS loading buffer, separated on a 10% SDS-polyacrylamide gel, and detected by western blotting and ECL⁵.

tionally, as expected, CBP binding was detected only in the presence of phosphorylated CREB. The composition of the protein complex at the HTLV-1 CRE was notably different (Fig. 4b). In this case, Tax associated with complexes containing only DNA and CREB in a manner that was independent of CBP. Consistent with the fluorescence assays, CBP associated with the HTLV-1 CRE:non-phosphorylated CREB complex in the presence of Tax. A model describing these interactions is depicted in Fig. 4c.

Our finding that Tax increases CREB binding specifically to the HTLV-1 CRE is consistent with previous observations^{10–12,18}. Because Tax does not alter CREB affinity for the consensus cellular CRE, it is unlikely that Tax functions primarily by increasing CREB dimerization¹⁹. If this was the case, Tax should increase the affinity of CREB for all CRE sequences. The degree to which induction of the HTLV-1 CRE depends upon the individual activation properties of Tax or CBP remains unknown, however, and both may contribute to the ability of Tax to activate the viral long terminal repeat. The inability of Tax to activate CRE-containing cellular genes has been puzzling. Our data show that Tax can indeed potentiate expression from the prototypical cellular CRE if the PKA pathway is also activated, presumably by recruiting Tax to the CRE:phosphorylated CREB:CBP (or p300) complex. These results suggest that Tax can stimulate the viral long terminal repeat under basal conditions but activate cellular

CRE-containing genes under conditions that lead to CREB phosphorylation. □

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Specificity of ribonucleoprotein interaction determined by RNA folding during complex formation

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MANY proteins involved in pre-mRNA processing contain one or more copies of a 70–90-amino-acid $\alpha\beta$ module called the ribonucleoprotein domain^{1–4}. RNA maturation depends on the specific recognition by ribonucleoproteins of RNA elements within pre-mRNAs and small nuclear RNAs. The human U1A protein binds an RNA hairpin during splicing^{5–8}, and regulates its own expression by binding an internal loop⁹ in the 3'-untranslated region of its pre-mRNA, preventing polyadenylation. Here we report the nuclear magnetic resonance structure of the complex between the regulatory element of the U1A 3'-untranslated region (UTR)^{9–11} and the U1A protein RNA-binding domain. Specific intermolecular recognition requires the interaction of the variable loops of the ribonucleoprotein domain with the well-structured helical regions of the RNA. Formation of the complex then orders the flexible RNA single-stranded loop against the protein β -sheet surface, and reorganizes the carboxy-terminal region of the protein to maximize surface complementarity and functional group recognition.

The RNA-binding domain of the human U1A protein, which comprises residues 2–102, including the amino-terminal ribonucleoprotein (RNP) domain, was complexed with an RNA construct containing one of two repeated binding sites from the polyadenylation regulatory element of the U1A pre-mRNA (Fig. 1). Samples were prepared with protein or RNA labelled with ¹⁵N or ¹⁵N/¹³C and mixed with the unlabelled counterpart. This allowed selective observation of the protein or RNA in the complex and the unambiguous identification of intermolecular nuclear Overhauser effect (NOE) interactions¹². This procedure

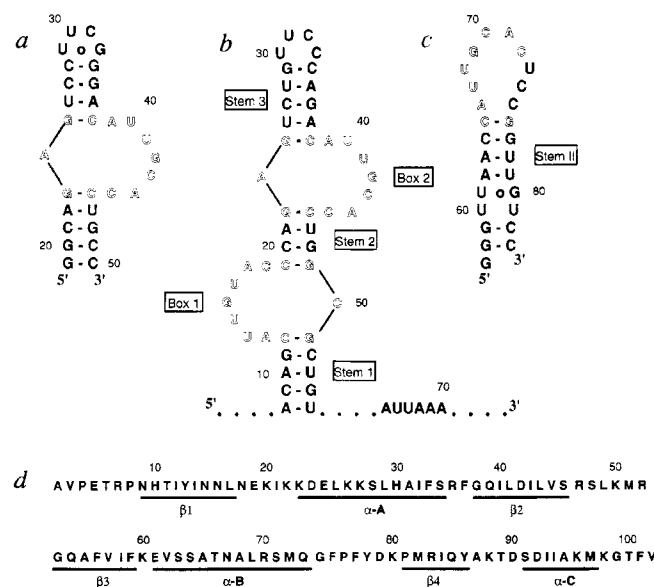
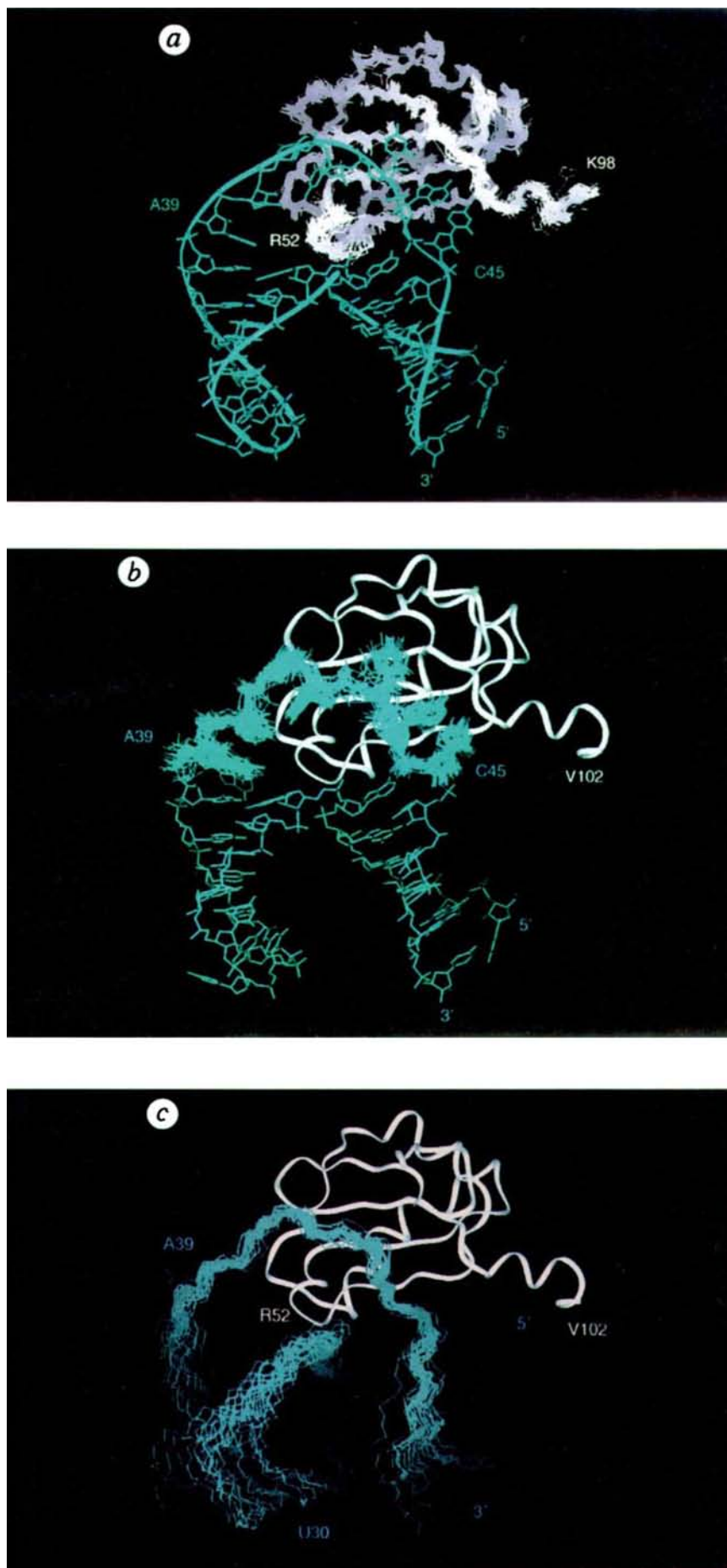


FIG. 1 a, Sequence and secondary structure of the RNA molecule used here; b, the complete polyadenylation regulatory element of the U1A pre-mRNA 3'-UTR; c, the related stem-loop II sequence from U1 small nuclear RNA, with the protein binding sites highlighted; and d, sequence and secondary structure of the U1A protein construct.

METHODS. Unlabelled and fully ¹⁵N or ¹³C/¹⁵N labelled RNA was synthesized by *in vitro* transcription with T7 RNA polymerase and purified as described^{17,26,28}. The RNA-binding domain of U1A (residues 2–102, as verified by mass spectrometry) contains two mutations of amino acids not directly involved in RNA recognition (Tyr31–His, Gln36–Arg; Fig. 1d)⁵. Native gel electrophoresis shows that this double mutant binds this RNA construct with $K_d < 10^{-9}$ M (ref. 17). Isotopically labelled protein was overexpressed in pET13a *Escherichia coli*²⁹ supplied with appropriate nutrients and purified as described². Spectral assignments were obtained from a variety of two- and three-dimensional experiments, including ¹H–¹³C–³¹P and ¹H–¹³C–¹⁵N triple-resonance spectroscopy. Spectra were recorded on Bruker AMX-500 or DMX-600 spectrometers equipped with triple-resonance gradient probes and processed using FELIX (Biosym, San Diego). Protein assignments are essentially complete (to be reported elsewhere), and RNA assignments complete with the exception of C5', H5' and H5'' and base NH₂ resonances within the internal loop region (C38 to C46, assigned only for the free RNA)¹⁷.

FIG. 2 Superpositions of all 25 converged structures for the complex. Average structures and superpositions were obtained using the program CLUSTERPOSE³⁰ and displayed with INSIGHT II (Biosym Technologies). Whenever a single structure is shown, it corresponds to the structure of lowest energy. *a*, Protein backbone atoms. *b*, RNA heavy atoms at the intermolecular interface. *c*, RNA backbone atoms. The r.m.s.d. for the protein is slightly better than that obtained for the free protein¹⁵, and the RNA loop is much better defined than for the free RNA¹⁷, owing to the disorder present in the latter case. The wider spread of the RNA backbone structures is reflected in a larger r.m.s.d. over the entire complex, and is caused by the absence of direct experimental information (or externally imposed non-experimental constraints) on the relative position of the two RNA helices. This reflects the short-range character of interactions detected by NMR, but also, probably, the presence of genuine conformational flexibility. The comparison between the present structure and the related hairpin crystal structure⁶ reveals an r.m.s.d. of 1.2 Å for the protein backbone. Differences between the two structures are in part attributable to the different RNA structural context but also to conformational flexibility at the RNA-protein interface in solution. Characterization of such dynamic processes will require further technical developments which are currently under investigation.



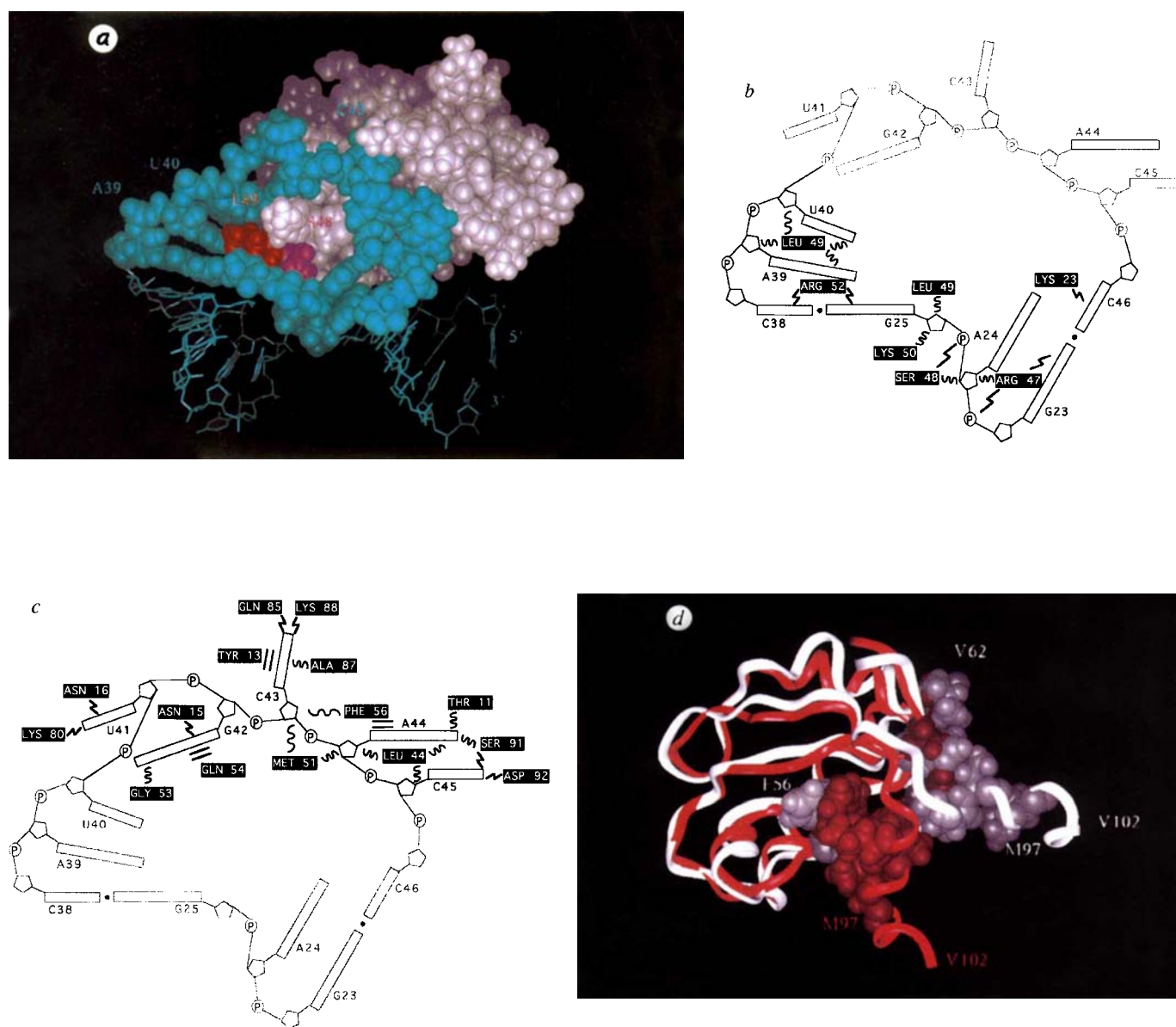


FIG. 3 *a*, Van der Waals CPK model representation of the structure highlighting the complementarity of the intermolecular surfaces; Leu 49 is red and Ser 48 is magenta. *b*, A first set of intermolecular contacts involve the interaction between exposed protein residues from the $\beta 2$ - $\beta 3$ and $\beta 1$ -helix A loops and well-structured elements of the RNA, including the G23-C46 and G25-C38 base pairs and the neighbouring A24, A39 and U40 residues. Intermolecular hydrogen-bonding and hydrophilic interactions are indicated by jagged lines, van der Waals and hydrophobic contacts by wavy lines, and stacking interactions are identified by parallel lines. All interactions shown in these diagrams are consistently observed in all converged structures and are defined by the extensive network of intra- and intermolecular NOE interactions. *c*, More extensive contacts involve the surface of the β -sheet and the $\beta 4$ -helix C loop and nucleotides U41 to C45. *d*, The protein conformational change reorients helix C away from the β -sheet surface; the hydrophobic residues stabilizing the free (red) and bound (white) protein structures are indicated by van der Waals spheres. *e*, Protein binding rearranges the RNA conformation. A positively charged patch on the protein (blue, left), corresponding to the $\beta 2$ - $\beta 3$ and $\beta 1$ -helix A loops, interacts with RNA (middle), dramatically changing the shape of the RNA molecule (right).

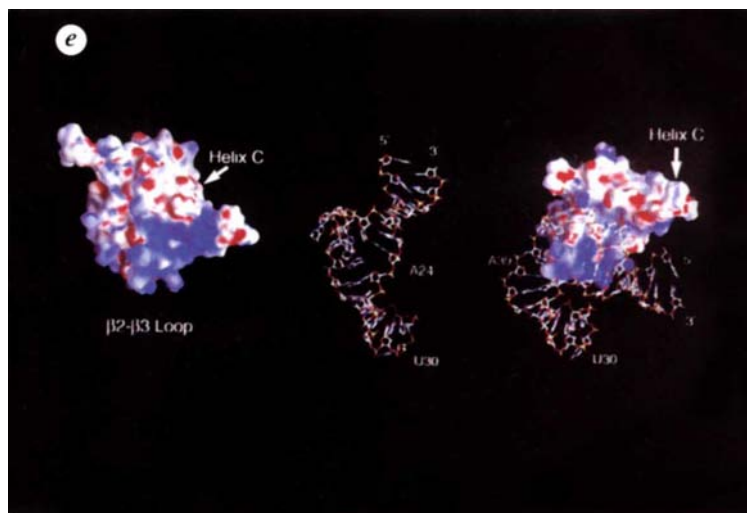


TABLE 1 NMR structure statistics

(a) NMR constraints		
Distance and dihedral angle constraints		
	Protein	RNA
Total	1,758	735
Intra-residue	565	357
Sequential	450	192
Medium/long range	722	51
Hydrogen bonds	21	25
Dihedral restraints	0	110
Protein–RNA distance restraints	96	
Total number of constraints	2,589	
(b) Structure statistics		
NOE violations		
Number >0.2 Å	17 ± 3	
Maximum violation	0.5 Å	
Angle violations		
Number >5°	1.6 ± 1	
Mean deviation from ideal covalent geometry		
Bond length	0.07 Å	
Bond angles	0.86°	
Improper	0.48°	
(c) R.m.s. deviations from mean structure (Å)		
Protein		
All ordered residues (7–98): backbone	0.61 ± 0.12	
heavy atoms	1.09 ± 0.13	
RNA (superposed on all heavy atoms for the indicated residues)		
All ordered regions (G20–C49)	2.59 ± 0.81	
Upper stem (G25–C38)	1.03 ± 0.37	
Lower stem (G20–G23, C46–C49)	0.80 ± 0.11	
Protein binding site (G23–G25, C38–C46)	1.29 ± 0.32	
Single stranded loop (A39–C45)	1.02 ± 0.16	
Protein–RNA (all heavy atoms for indicated residues)		
All ordered regions (7–98, G20–C49)	2.52 ± 0.73	
Complex interface	1.36 ± 0.28	

Interproton distance constraints were obtained from nuclear Overhauser effect spectroscopy (NOESY) spectra recorded in H₂O and in D₂O at mixing times of 40, 80, 100 and 120 ms, and from ¹³C- and ¹⁵N-separated three-dimensional NOESY spectra recorded at 100 ms mixing time with labelled RNA or protein and unlabelled counterparts. NOE cross-peaks were subdivided into four classes by comparison with cross-peaks corresponding to known distances (RNA H5–H6 peaks and protein sequential $d_{\alpha\alpha}$ and helical $d_{\alpha\alpha}(i, i+3)$ peaks): strong (0–2.5 Å), medium (0–3 Å), weak (0–4 Å), very weak (0–5 Å); lower bounds were not used. Peaks present only at 100–120 ms mixing time or resolved only in the three-dimensional spectra were only given for 5- or 7-Å upper bounds to reduce potential errors resulting from spin diffusion. Most NOE cross-peaks involving exchangeable RNA protons were only classified as very weak, but U NH to A H2 and G NH to C NH₂ interactions in Watson–Crick base pairs were included as weak constraints. Hydrogen bonds in the RNA were identified by the observation of a significant downfield ¹H shift and a slow rate of exchange with solvent; the constraints were used for G·C and two for A·U base pairs. Although both U40 and U41 NH would satisfy the above criteria, hydrogen-bonding constraints were not introduced at the RNA–protein interface to avoid misidentification of acceptor atoms. For the protein, 21 very slowly exchangeable amide resonances were observed in spectra of the complex in D₂O, and hydrogen-bonding constraints were introduced by analogy with the free protein structure¹⁷. No non-experimental constraints were added to maintain idealized A-form RNA helices or perfect base-pair planarity. Dihedral angles in the stem regions were constrained by analogy with the free RNA wherever all chemical shifts were essentially identical between the free and bound RNA (G19–A22, U26–A38, U47–C50). Typical uncertainties on the dihedral angle constraints were ±120° for α and ζ and ±40° or ±60° for all other dihedral angles. The sugar conformations of U31, C33, U41 and C43 were constrained as C2'-endo from correlated experiments; all other residues were constrained in C3'-endo except G42, for which an unambiguous sugar conformation could not be obtained. Structure calculations were performed with X-PLOR²⁹, using only the experimental information just described, RNA and protein stereochemistry and van der Waals interactions; electrostatic and dihedral components of the force field were inactive throughout to minimise any bias. We subjected 75 starting structures with completely random RNA and protein torsion angles to

yielded an unusually complete experimental constraint list (Table 1), leading to a well-defined structure, without non-experimental assumptions about the RNA or protein structures or the intermolecular interface (Fig. 2a–c).

The 3'-UTR RNA contacts the U1A N-terminal domain on the surface of the four-stranded β -sheet and in three variable loops (β 1-helix A, β 2– β 3, and β 4-helix C). The β 2– β 3 loop protrudes through the hole in the RNA defined by the single-stranded nucleotides A39 to C45, and A24 and the two base pairs closing the loop G25·C38 and G23·C46 (Fig. 3a). The RNA structure is severely kinked, and the single-stranded nucleotides are splayed out across the surface of the β -sheet. Despite the absence of intramolecular hydrogen bonds within the internal loop, each of the unpaired nucleotides is involved in intra- and/or intermolecular stacking interactions, as observed in the crystal structure of the related U1A–hairpin complex⁵. As expected from the similarity in sequence, intermolecular interactions between residues A39 to C45 and the U1A protein are similar for the two complexes, but interactions observed in the present structure involving the β 2– β 3 and β 1-helix A loops and G23, A24 and C46 have no counterpart in that crystal structure.

The structure of the interface suggests that the RNA–protein contacts can be divided into two sets. A first set of interactions involves residues from the β 2– β 3 and β 1-helix A loops and seven nucleotides from the RNA, these being the G25·C38 and G23·C46 base pairs, A24, A39 and U40 (Fig. 3b). A24 interacts with Ser 48 and stacks on the G23·C46 base pair, and these three nucleotides interact with Arg 47 and Lys 23. Similarly, A39 stacks on U40 and on the G25·C38 base pair; these nucleotides interact with Arg 52 and form extensive hydrophobic contacts with Leu 49 (Fig. 3a). The importance of Leu 49 in RNA recognition by U1A has recently been emphasized¹³. These interactions are reinforced by electrostatic interactions between the β 1-helix A loop and the phosphodiester backbone. The second set of interactions involves residues from the β -sheet surface and the β 4-helix C loop and nucleotides U41 to C45 (Fig. 3c). Among these contacts are three intermolecular stacking interactions involving highly conserved amino acids: G42 on Gln 54, C43 on Tyr 13 and A44 on Phe 56.

The region C-terminal to the RNP domain is critical for RNA recognition in several members of this protein family, including U1A^{3,5,7}, and heterogeneous nuclear RNP C¹⁴ and U1 70K¹. Amino acids 92–98 form a helix in the solution structure of the free U1A protein^{8,15}, which is absent in shorter constructs (residues 2–95)^{2,3}. Highly conserved hydrophobic residues located on the surface of the β -sheet are buried under this helix in the free protein, forming a small hydrophobic core with Ile 93, Ile 94 and Met 97 (Fig. 3d, red). The C-terminal helix may stabilize the

restrained molecular dynamics. No assumptions were made at any stage about RNA or protein structures, and intermolecular 'docking' steps were never introduced. The free protein¹⁷ and RNA structures¹⁹ and the structure of the complex were calculated instead using essentially the same four-step protocol: (1) simulated annealing used distance constraints to fold the complex (70 ps dynamics run of 10,000 steps at a temperature of 1,000 K); (2) refinement (30 ps dynamics run of 30,000 steps at 1,000 K), then slow cooling to 300 K; (3) further refinement, gradually introducing RNA dihedral angle constraints; and (4) minimization. Non-converged structures were rejected, based on the analysis of the total pseudoenergy of NOE violations. Ensembles were constructed by adding individual structures in increasing order of NOE energy, and the r.m.s.d. of the ensemble recalculated at each step. Structures were retained from plateau regions of the resulting profiles of r.m.s.d. against ensemble size; structures with significantly higher NOE energy, lying beyond such plateaus, were discarded. This procedure resulted in retention of ~40% of all RNA structures and ~70% of all protein structures in the complex. Overall, 25 of 75 starting structures displayed satisfactory violations of all experimental constraints. This rate of convergence is lower than for protein structures¹⁷ but typical for RNA^{19,26,30}.

domain¹⁶ through these interactions, but the β -sheet surface must become accessible for RNA recognition. To allow this, helix C rotates away from the β -sheet (like a 'cat-flap'), thereby forming an alternative core involving His 10, Leu 41, Ile 58 and Val 62 (Fig. 3d, white) and residues from helix C (Ile 93 and Ile 94). This repositioning of helix C buries the 'alternative' hydrophobic patch, exposes the β -sheet for interaction with the RNA, and presents the β 4-helix C loop to the RNA. Mutant proteins lacking helix C would be unable to form the alternative hydrophobic core, and this could account for the ~ 30 -fold decrease in affinity for mutants containing a shortened helix C (for example, the $\Delta 96$ –102 truncation) and the much more severe effects of mutants truncated at Ser 91 (refs 2, 7).

Protein binding orders the RNA loop and changes the overall shape of the RNA (Fig. 3e). This rearrangement is best described in terms of the stacking pattern of the bases in the internal loop. In the free RNA¹⁷, the stacking pattern is C38:A39:U40:U41, G42, C43, A44:C45:C46, where a colon denotes stacking. In the bound RNA, the stacking pattern is C38:A39:U40, U41:G42:Gln 54, C43:Tyr 13, Phe 56:A44:C45, C46, where intermolecular interactions are also included. Thus, in both the free and bound RNA, the 5'-end of the internal loop forms a continuation of the helical stem. Nucleotides U41 to C45 are flexible in the free RNA¹⁷, but the interaction with the protein orders this region of the RNA. The surface of the β -sheet should therefore be seen not only as an RNA-binding surface, but also as an RNA-folding platform. The three protein residues that stack on RNA bases are highly conserved among RNP proteins (Gln 54 is Tyr or Phe in many RNP proteins), suggesting that this ordering process may be a general feature of RNP–RNA recognition.

Our results point to an unexpected recognition mechanism. First, the variable loops of the RNP domain interact with the well-structured helical regions of the RNA. Second, folding of the flexible, RNA single-stranded loop and reorganization of the C-terminal region of the protein domain maximizes surface complementarity and functional group recognition. This mechanism explains both the requirement for RNA structural integrity for U1A binding and the ability of U1A to recognize the identity of the single-stranded nucleotides. The role of RNA structure and plasticity illustrated by this mechanism may explain how tight

binding ($K_d \approx 10^{-11}$ M) and high specificity can be achieved with a relatively small intermolecular surface area ($2,300 \text{ \AA}^2$). Seen in this context, intermolecular contacts between the U1A RNA-binding domain and its substrate fall into two categories. One subset of contacts involves a region of positive electrostatic potential encompassing the β 1-helix A and β 2– β 3 loops, the sequence of which is unique to the U1A protein (Fig. 3b). These residues participate in the 'rigid body' interaction with the Watson–Crick base pairs closing the internal loop and the well-stacked nucleotides in the internal loop. The second set of contacts occur by 'induced fit' and involve residues on the surface of the β -sheet and the β 4-helix C loop (Fig. 3c). These contacts cannot occur without ordering residues U41 to C45 and reorienting helix C (Fig. 3d, e). The protein structural change exposes the hydrophobic surface of the β -sheet to the RNA bases, and allows stacking and hydrophobic interactions with residues U41 to C45, while stabilizing an extensive hydrogen-bonding network involving amino acids on the β -sheet surface and the β 4-helix C loop.

In several DNA–protein complexes, the DNA double helix provides a folding surface for the protein in specific binding events¹⁸. In the present system, the protein β -sheet provides a folding platform for the nucleic acid component. This situation has similarities with the mechanism of recognition of the DNA minor groove by the TATA-box-binding protein^{19,20} and high-mobility-group proteins^{21,22}, and may have analogous functional implications. In the full complex between U1A and its pre-mRNA, two protein monomers bind the full RNA regulatory element (Fig. 1b) and inhibit polyadenylation by a direct interaction with poly(A) polymerase^{9–11}. Protein binding kinks and stiffens the RNA structure, and this could be a general mechanism to bring distant RNA regulatory elements close together. RNA reorganization may be a common feature of recognition by RNA-binding proteins, including viral transactivators^{23,24} and protein cofactors of ribozymes²⁵. Furthermore, many ribosomal proteins have the same structure as RNP proteins²⁶, suggesting a potential role for protein-induced RNA reorganization in ribosome assembly. It will be interesting to investigate the dynamic properties of these structural changes and study how these processes contribute to intermolecular recognition. □

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CORRESPONDENCE and requests for materials should be addressed to G.V. (e-mail: gvl@mrc-lmb.cam.ac.uk). Coordinates have been deposited in the Brookhaven Protein Data Bank, accession number 1NUM.

An analytical perspective

Supporting a hypothesis requires reliable data and, of course, sound equipment to produce it — this issue features a system for trace analysis, a new NMR probe, instrument software and an electrospray mass spectrometer.

THERMEDICS DETECTION has developed the Flash-2D-GC **high-speed gas chromatography (GC) system**. It is a recent addition to the company's line of trace-detection products, including the EGIS explosives-detection system and the Alexis system for detecting contaminants in refillable bottles. The system is designed to analyse a sample quickly and can also perform analyses that typically require several instruments, according to the manufacturer. The key technology is the flash temperature programming. There are six independent temperature-programmable zones, and as many as four temperature-programmed cold spots can be used for high-speed injection and/or purge-and-trap procedures. Effective cold-spot heating speeds of $6,000\text{ }^{\circ}\text{C s}^{-1}$ can be achieved using digital electronic control. Reproducible, high-speed separations can be performed using two independent columns, whereas GC-column temperature programming can achieve heating speeds as high as $100\text{ }^{\circ}\text{C s}^{-1}$.

Reader Enquiry No. 100

GATC has developed an **automated membrane developing system** to analyse membrane-bound biomolecules automatically. Computer-controlled protocols and automatic scanning are designed to eliminate all manual steps. The system works with all available membranes and offers standard protocols for the analysis of DBE sequencing membranes, Southern, northern, western and dot blots. The membrane carrying the biomolecules is placed onto the outer surface of a transparent cylinder, which slowly rolls through a low-volume bath containing the reagents. A thin film of reagent is sufficient for optimal signals. Therefore, membranes as large as $30\text{ cm} \times 50\text{ cm}$ may be developed with a minimum of reagents (15–20 ml). A computer controls the temperature, as well as the entire detection protocol (reagents are automatically pipetted from the reagent storage rack into the incubation bath). A built-in visible/fluorescent scanner digitizes the result and stores the image on a 1-gigabyte internal hard disk. For further processing, data may be transferred electronically through an integrated network port or by floppy disk.

Reader Enquiry No. 101

Instrument assortment

Malvern Instruments now offers the Mastersizer **diffraction autosampler**, which is designed for use on its range of instruments that use the technique of laser diffraction to determine particle size. The autosampler allows users to queue up samples for measurement and automatic processing. The company also offers information on new developments



Malvern Instruments' new autosampler.

in the area of regulatory compliance, particularly pertinent to the pharmaceutical industry. In addition, products covering submicron particle characterization and the monitoring of ultrapure liquids are also available.

Reader Enquiry No. 102

Hewlett-Packard and Bruker jointly offer the Esquire **electrospray ionization (ESI) ion-trap mass spectrometer**. The spectrophotometer will be available with the HP 1100 high-performance liquid chromatograph (HPLC), the HP 3D capillary electrophoresis (CE) system and ChemStation software. Because Esquire and the HP 1100 Series HPLC (or the HP 3D CE system) are controlled by a single personal computer, researchers can now perform qualitative structural confirmations of synthetic products and biomolecules using an integrated liquid chromatograph/ion-trap mass spectrometer/mass spectrometer (LC/MS/MS) system. The nonlinear, multipole ion trap is said to provide fast ejection of ions for improved resolution and improved mass accuracy. It has a $6,000\text{ }m/q$ mass range for the analysis of large proteins, non-covalently bonded compounds, glycoproteins and other large molecules. A NanoSpray option is also

offered for the analysis of limited volumes of samples. Extended MS/MS experiments can be performed on as little as one to two microlitres of total sample at flow rates of 25 nl min^{-1} .

Reader Enquiry No. 103

The new CM120 BioTwin **transmission electron microscope** from Philips Electron Optics has been developed for low-temperature work using a high-contrast imaging system and enhanced analytical performance. The newly designed objective lens is said to offer a high-contrast image of thick and inherently low-contrast specimens, a long focal length and a high spherical aberration constant. According to the manufacturer, the magnification range is optimized for life science applications, with a very low magnification in the high contrast imaging mode. The specimen environment is surrounded by large surface cryoshields that ensure a clean vacuum, and hours of contamination-free observation of cooled and uncooled specimens. In addition, the CompuStage eucentric side-entry goniometer is said to



BioTwin from Philips Electron Optics.

provide high stability and allow precise specimen movements and accurate recall at low temperatures. This is important for stereo imaging and automatic X-ray detection. High count rates and fast X-ray mapping are assisted by the EDX detector. This can be brought close to the specimen, thus permitting a high solid angle for X-ray detection.

Reader Enquiry No. 104

Chromacol's SCI-VI vial system offers one microvial, in 100- μ l, 200- μ l or 300- μ l sizes, to be used with a number of support sleeves that can accommodate a variety of instruments. For example, Chromacol's glass support sleeve (SV-S11G) plus the new 100 μ l crimp-top vial (01-CVG) can be used with Hewlett Packard's 7673A autosampler for GC analysis. The 01-CVG by itself may be used in the Perkin Elmer ISS200 liquid chromatograph, a Merck-Hitachi autosampler or a Gilson autosampler. The vial can then be recombined with the glass support sleeve and for use with the Shimadzu AOC 1400 GC autosampler.



Reader Enquiry No. 110

Varian has introduced the SuperProton NMR probe (detector) as the first of the company's new NMR probes that employs high-temperature superconductor technology to increase probe sensitivity. According to the manufacturer, this technology produces an increase in sensitivity and will increase the range of problems that can be solved by NMR. The probe utilizes RF coils made of a high-temperature superconductor, YBCO. It is said to allow for analysis of smaller quantities, such as those found in by-product and metabolite analysis.

Reader Enquiry No. 105

The electrochemical flow-through trace analyser (EFTA) from Metrohm is an extension of the existing 693/694 trace analyser. It is composed of a flow-through cell, which is mounted in the 694 VA stand and a 708 sampling/pumping station. The sample is introduced into an eluent stream by the station, it is then pumped over the electrode assembly where the ions of concern are preconcentrated on the working electrode. After flushing with a suitable eluent, the flow is stopped and the ions are stripped back into solution and a voltammogram is recorded. The operation process is controlled by the 693 VA Processor. This system uses a simplified design so that trace analysis can be carried out by non-skilled personnel.

Reader Enquiry No. 106

Software supply

MultiChrom 2.2, a multiuser chromatography data-handling system developed by FI Labsystems, now offers spreadsheet facilities, multiple headspace extraction to allow determination of volatile materials emitted by solids, and facilities for constructing calibration curves using added standards. The system is also useful for importing LIMS sample information from text files, rapid system configuration data change across a whole file and adding new instrument control options. The system runs on the DEC Vax platform under the open VMS opera-

tional system, and will shortly be available on the DEC Alpha AXP.

Reader Enquiry No. 107

National Instruments has released a free multimedia demonstration of Measure, an Excel software package for data acquisition and instrument-control in a Windows environment. Also available is a new four-page, full-colour brochure, which highlights sample screens of the easy-to-use, interactive menus for controlling plug-in data acquisition boards and serial measurement devices. It describes how spreadsheet users can use this software to improve productivity and even automate experiments with Excel macros. The demonstration includes various examples of the data-acquisition capabilities, report generation and on-line documentation.

Reader Enquiry No. 108



Measure software.

Hitachi Instruments has introduced UV-Visions instrument control software for their new U-2010 and U-3010 ultraviolet/visible spectrophotometers. Based on the Windows 95 or Windows NT platforms, the software is designed to simplify operation, improve productivity and maximize information output. This multitasking software is said to allow foreground and background operation of multiple software packages, as well as post-run processing while the analyses continue. The post-run processing, with in-place OLE activation, is said to enhance spectral presentation and provide a range of arithmetic and data management functions. The instrument control package includes quantitative analysis, wavelength and time scans, wavelength programming and multi-component analysis. The U-2010 features double-beam stability for improved accuracy and repeatability over time, a 100-mm beam separation using a half-mirror to

eliminate moving parts and a compact footprint. The U-3010 utilizes the low stray light, variable bandpass, high energy and linear absorbance greater than three.

Reader Enquiry No. 109

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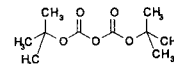
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